

**EFFECTS OF HIGH PRESSURE OF GASES
ON MICROBIAL PROCESSES**

Sven-Olof Enfors

Chemical Centre
Technical Microbiology
University of Lund

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PREFACE

The origin of this work was a 3 year-scholarship (67-636-S) which I was awarded by the Swedish Board for technical development (STU) in 1968. The project has received additional support from the Swedish Foundation for Applied Microbiological Research (STMF) since 1973. During 1968-1974 the work was performed at Karolinska Institutet, to start with in the Bacteriological Biotechnology Research Group and later on in the Department of Applied Microbiology. Since July 1974 the work was continued at the Chemical Center of Lund University.

The project was initiated by Professor Carl-Göran Hedén and I am very grateful to him for introducing me to such a fascinating topic. Since then, the work has been continued under Professor Nils Molin's supervision. I am sincerely grateful to him for the very good atmosphere for free research activities that he has created within the limits of applied research. This freedom and Professor Molin's enthusiastic support have been of utmost importance during this period. I am also indebted to all my colleagues at ATM for their contribution to a stimulating research atmosphere.

During the various phases of this work I have had very good technical assistance from Ulla Darenius, Gull-Britt Larsson, Gunilla Wilhelmsson and Katarina Åkesson. For the assistance in the design and construction of much of the equipment my thanks goes to Henry Sundberg and Björn Seger. During the design and construction of the pressure fermenter I had good and stimulating cooperation with Chemoferm AB. I also want to thank Anna Paulsson and Margareta Nelke for the typing of the manuscript.

INTRODUCTION

Microorganisms are, for their growth, dependent on a water based medium in which their nutrients are dissolved or sometimes just dispersed. As for solids like salts, proteins, carbohydrates, or the like, these can mostly be dissolved in the water phase to those concentrations that are desired for experiments, and the knowledge about the influence of these components on the metabolism and physiology of microorganisms is therefore considerable. Many microorganisms are for their metabolism dependent also on gaseous components like oxygen, nitrogen, carbon dioxide, methane, hydrogen, and so on. It is only the water dissolved part of these gases which can be used by the organisms. The solubility of the mentioned gases in water is at normal pressure and temperature very low, in the order of 10 mg/l. Knowledge about the effects of higher concentrations of these components on the metabolism and physiology of microorganisms is, because of that, relatively limited.

The increased solubility of a gas in water, that occurs when the partial pressure is increased above 1 atm, will give concentrations of dissolved gases that cells are seldom exposed to in nature. This might cause effects on metabolism which are of a similar type as those caused by increased concentration of solid nutrients. In other words, one might expect activating as well as inhibiting effects from an increased pressure of a gaseous nutrient. For instance, it is reasonable to suppose that the growth kinetics of methane oxidizing bacteria at methane limited growth depends on the concentration of methane in the same way as the growth of other bacteria depends on, for instance, the concentration of carbohydrates at carbohydrate limited growth. This kinetic relation can be described by the Michaelis - Menten model. The saturation constant (or Michaelis' constant), i.e. the concentration of the growth limiting substrate component at which the growth rate will be half the maximum growth rate, is for carbohydrates in the order 10 mg/l. In the common method to cultivate methane oxidizing bacteria a 50-50 mixture of methane and air is used. Then the solubility of methane in water is about 7 mg/l. If one supposes that the saturation

constant for methane in this case has the same order of magnitude as was mentioned for carbohydrates, then a 50-50 mixture of methane and air would not give a high enough methane concentration to yield maximum growth rate. A similar discussion can be developed concerning the effects of increased pressure of other gases on the growth rate of microorganisms. In the case of oxygen, however, which is the most well documented of these gases, there are many contradictory figures reported in literature.

The first task in this thesis was to investigate how the growth rate of bacteria, particularly methane oxidizing bacteria, responded to an increased gas pressure over the culture. The investigation constituted one of the approaches, made in a project on single cell protein.

When a microbial culture is growing fast it will consume some of the nutrients at a high rate. Most nutrient components can be added to the medium at the beginning of the process or at intervals during the process. Gases, however, have so low solubility that they will be consumed very quickly (in seconds or minutes) if they are not replenished continuously. A large and well known problem in fermentation technology is to achieve efficient oxygen transfer rate. For a rapidly growing methane oxidizing culture with high cell density, a combination which is required for an economically valid production of single cell protein from methane, the methane transfer rate will be another critical process parameter. Since oxygen in that case competes with methane for space in the gas phase, and the oxygen demand in this process is extremely high, the gas transfer problems in this process can be expected to be a major problem. The gas transfer rate in a fermenter where bacteria have decreased the concentration of dissolved gas to zero will be directly proportional to the solubility of the gas. The solubility of the gas is proportional to the partial pressure according to Henry's law. Thus, by cultivation under increased gas pressure it should be possible to increase the gas transfer rate. However, this pressure will not act selectively on one gas, which means that for instance the carbon dioxide concentration in the medium will increase, the consequence of which is difficult to judge. The increased concentration of dissolved nitrogen and the hydrostatic pressure are other parameters to be taken into account.

A fermenter design project was included as a second part of this thesis. The use of increased gas pressure is a tempting approach to the gas transfer problems in many processes but there has been a lack of suitable instruments for this work. When a process is to be studied under elevated air pressure it is important that all the other process parameters are kept under the same control as in the original process. This means that one needs a fully equipped high pressure fermenter, which allows for all the analyses and control operations which are used in the ordinary fermenter.

Litterature studies disclose that the oxygen concentration will be critical for growth when it decreases below a level corresponding to a few per cent of oxygen in the gas phase. There also exists an upper limit of concentration where oxygen becomes toxic. The reported data on this are somewhat diverging and it is true that different biological reactions show varying response to high oxygen concentration. For instance, microorganisms do not have the same sensitivity to high oxygen pressure throughout their life cycle, a fact that has been used for synchronization of cell division.

A third task in this thesis concerns an investigation of the effect of high air pressure on bacterial sporulation. Special attention was payed to the possibility to synchronize the sporulation process in the same way as cell division can be synchroni ed by high oxygen pressure. The sporulation of a culture is mostly extended over a period of many hours or days, but the process in one organism takes considerably shorter time. If a simultaneous sporulation can be achieved, this is of great value for the biochemical studies of the sporulation process.

A similar discussion can also be made about the possibility to stop and synchronize the germination of a spore. The fourth task of this work was to investigate the effect of increased air pressure on the germination process. The results of this showed, however, that the inhibition of germination by high air pressure, that was found in an early part of this investigation, was not a concequence of the high oxygen pressure and the chemical reactivity that comes from this. This work was therefore extended to the effects of nitrogen and hydrostatic pressure and finally to other gases, among then the noble gases.

The literature concerning the effects of high gas pressure on biological systems is spread over a wide area of disciplines: Physiology, anaesthesiology, experimental biology, microbiology, biochemistry, molecular biology, oceanography, aerospace medicine, and so on. Since some of these disciplines do not belong to those covered by the microbiologist in the daily literature research I have considered it important to give a relatively large literature background in this thesis. When results emerged on the effects of high gas pressures and they had to be explained, it soon became evident that a basic knowledge on the effects of hydrostatic pressure was important since the hydrostatic pressure cannot be excluded in experiments with high gas pressure. Therefore the literature review also includes a part on hydrostatic pressure effects on biological systems.

EFFECTS OF HIGH PRESSURE ON BIOLOGICAL SYSTEMS. A REVIEW.

Introduction

During the last years biological systems under high pressure have attended an increased interest. It is especially in the investigation of the deep sea resources and the possibilities to exploit them that the pressure has become a parameter of the same fundamental importance as temperature and pH. The interest for high pressure, especially of gases, has also gained interest in space research. Further more pressure studies have proved to be a valuable tool in the studies on the kinetics of biological reactions. The effects of high pressure on the solubilities of gases, from the biotechnological point of view, has made this parameter interesting. Several gases which are normally considered to be inert in biological reactions have been shown to be able to interfere with the cellular reactions if the cells are exposed to high enough concentrations of the dissolved gas. This can be achieved by increasing the pressure of the gas phase.

This literature review has been divided into two parts. The first part deals with the effects of high hydrostatic pressure and the second deals with the effects of high gas pressure. During work with biological systems under high gas pressure there is always an equally high hydrostatic pressure. This fact makes it necessary to have a basic knowledge about hydrostatic pressure effects on biological reactions when discussing the effects of high gas pressure.

Effects of high hydrostatic pressure

History. The interest in effects of high hydrostatic pressure on living organisms originates from the last part of the 19th century when the Challenger expedition (1872-1876) and the Talisman expedition (1882-1883) detected living organisms in the deep sea down to 6 000 meters. The compression in the sea amounts to approximately 1 atm per 10 meters (more exactly 0.99 - 1.05 atm depending on temperature, compression, salt content and latitude).

This means that organisms studied by these expeditions had survived and maybe lived at 600 atm pressure. The first publications on experiments with living cells at high pressure were made during the years after the Talisman expedition by Regnard in France (1, 2).

It was soon revealed that some life processes were retarded, some were accelerated and some were not influenced at all by the pressure. Very high pressures, in the range of 5 000 atm were found to denature proteins (3) and this was generalized to the hypothesis that high pressure is injurious to cells because of protein denaturation. The complicated dependence on temperature had not been detected during these first decades of high pressure research.

During the 1930's and the 1940's a large amount of deep sea organisms were studied with respect to their viability at pressures up to 1 000 atm. It was found that many species, especially amongst the more primitive organisms were able to survive at 1 000 atm. These organisms were mostly typical deep sea species while the organisms living closer to the surface were inactivated or killed by a few hundred atm. One of the fundamental cellular reactions that was studied was the cytoplasmic sol-gel equilibrium which was very sensitive to pressure. This equilibrium plays an important role in the cell division and the movement of protozoa (4).

Until the 1940's the effects of pressure on biological systems were studied without the basic knowledge about the way in which pressure influences the kinetics of chemical reactions. A corresponding theory of the temperature effect on the kinetics had been developed by Arrhenius in the late 19th century. Eyrings theory on the absolute reaction rate (5) formed the basis for the understanding of the influence of pressure on biological reactions. The explanation of this theory needs considerable space but a short summary of the principle will be given here:

"Any chemical reaction passes via the formation of an intermediate unstable compound a so called activated complex, which quickly (half time 10^{-13} sec) decomposes into the final products or returns to the original state. A change in volume ($\Delta V^{\ddagger}$) is involved in the formation of the activated complex

and this change can be positive, negative or zero. The pressure dependence of the reaction rate of the complete reaction (the formation of the final products) is dependent on ΔV^x according to

$$k_p = k_o \cdot e^{-p \Delta V^x / RT}$$

in which k_p and k_o are the rate constants at pressure p and zero respectively and RT is the product of the general gas constant and the absolute temperature.

The equation above can be rearranged to

$$\log k_p = - \frac{\Delta V^x}{2.3RT} \cdot p$$

which shows that the reaction rate (k_p) will increase, decrease or be unaffected when pressure is raised depending on whether the formation of the activated complex involves a volume decrease ($\Delta V^x < 0$), a volume increase ($\Delta V^x > 0$) or no volume change at all ($\Delta V^x = 0$).

By measuring the reaction rate at different pressures ΔV^x can easily be calculated. A series of measurements including a discussion of the theory have been published by Stearn and Eyring (6).

Physical-chemical effects of high pressure. When the pressure is increased to thousands of atm the distances between and within the molecules are changed and this can sometimes cause phase transitions. From the biological point of view such phase transitions are of interest mainly in the case of water. At temperatures between -22°C and -35°C water is normally in phase ice (I). When the pressure is increased to 2 100 - 3 500 atm ice (I) will turn into ice (III), a reaction that is accompanied by an approximately 20% volume decrease. This phenomenon can be used for disintegration of cells (7, 8).

The thermal conductivity of water increases when the pressure is increased and it is about 6% higher at 1 000 atm than at 1 atm and 30°C . The viscosity of water is also pressure dependent. At low temperatures ($0 - 10^\circ \text{C}$) the viscosity decreases with increasing pressure up to 1 000 atm and

increases again if the pressure is raised still more. At higher temperatures than 10°C the viscosity of water increases with the pressure also at the lower pressures. At constant pressure the viscosity decreases when the temperature is increased (9).

From the biological stand point the most obvious physical-chemical effects of pressure are the above mentioned effects on the reaction rate but also the effect on the chemical equilibrium. Le Châtelier's principle says that the equilibrium of a chemical reaction will be adjusted towards the side with the smaller volume when the pressure is increased. Consequently the dissociation of acids and bases will increase at compression. The pH of a water solution will thus be pressure dependent. A phosphate buffer with pH 7.0 at 1 atm will change to pH 6.6 when compressed to 680 atm (10). The molar volume change of the dissociation of H_2PO_4^- is -24 cm^3 . As for biological macro-molecules like enzymes the molar volume changes can be in the order of -100 cm^3 , a fact that explains why such molecules can be especially sensitive to pressure changes (11).

The molecular reorganization of proteins which follows as a consequence of increased ionization and decreased molar volumes may cause the protein to denature. The pressure required for this is often in the range of thousands of atm but even lower pressurization may affect the biological function of the protein.

Pressure effects on proteins. The stability of a protein is determined by many types of bondings. The stronger covalent bondings require large amounts of energy for breakage and mostly these are not changed during the denaturation of a protein. Ion bondings are pH dependent and will therefore change with respect to number and character when pH is changed. Large pH variations often cause denaturation which means that there is mostly a pH optimum inbetween the extremes. The weaker hydrogen bonds and the hydrophobe bonds (van der Waals forces between nonpolar groups) can easily be broken and reformed in other places in the molecule when it is exposed to structural changes. Also these kinds of changes can denature a protein. The process of denaturation is often, but not always, irreversible.

A partial and reversible denaturation can be achieved when the protein is exposed to moderate pressure or temperature. There are many denaturing agents: organic solvents, acids and bases (pH changes) and salts (high ion strength). To these can be added physical agents like irradiation, heat and pressure.

During the 1930's and the 1940's a series of experiments were carried out which clearly showed that the effects of pressure on the enzyme activity (and the organism) cannot be studied unless the temperature is regarded. It was especially the work with the pressure dependence of the luminiscence that revealed this. The luminiscence intensity (luciferase activity) of a bacterial culture was measured during varying pressure-temperature conditions. At low temperatures the enzyme activity decreased with increasing pressure (to 400 atm) but at temperatures above optimum at 1 atm (25° C) the activity increased with increasing pressure (12). A similar relation has been shown in asparatase from Escherichia coli (13).

In order to understand these circumstances one has to regard the temperature effect on the enzyme activity. In the reaction where the product (P) is formed from the substrate (S) there is an activated complex (ES) between the enzyme (E) and the substrate:



When temperature is increased the reactants are furnished with kinetic energy and this will increase the complex formation rate (k_1). The complex ES will return to S + E with the rate k_{-1} or form P + E with the rate k_2 . The higher the concentration of ES, the more products will be formed per time unit. In short, the product formation rate increases with temperature. However, this is valid only as long as the temperature is below the temperature optimum of the enzyme because of another temperature depending equilibrium which gains importance at high temperatures. Increased temperature also furnish the enzyme with kinetic energy and at superoptimum temperatures this leads to an increasing reorganization of the structure - heat denaturation. This denaturation is reversible as long as the heat supply is not too extreme. At temperatures below the optimum this reaction does not effect the formation of ES.

Heat denaturation of the protein is a volume increasing process and this is counteracted by increased pressure. This explains why increased pressure has an activating effect on enzyme reactions at superoptimum temperatures. At optimum and suboptimum temperatures the increased pressure depresses the enzyme activity because of its gradual reorganization (pressure denaturation) of the molecule. One can also say that increased temperature reverses the pressure denaturation at suboptimum temperatures.

The contrary effects of heat and pressure at superoptimum temperatures can make an enzyme active at 105°C and 1700 atm (14). These circumstances are sometimes valid also for complete organisms. Bacteria have been able to reproduce at 104°C at a pressure of 1 000 atm (15).

The pressure and temperature ranges that were covered by the discussion above are 0 - 1 000 atm and 0 - 100°C i.e. approximately the conditions in which life can exist on earth. At higher pressures many enzymes are quickly denatured while others are stable. Since these conditions do not prevail in the biosphere they have been excluded from this discussion.

Pressure effects on nucleic acids. There are few investigations on effects of pressure on nucleic acids. It is mainly DNA that has been studied and the results show that DNA is much more stable than proteins at high pressure. Most proteins denature by a few thousand atm but DNA can withstand 10 000 atm without denaturation. In an investigation it was shown that the transforming capability of DNA from Bacillus subtilis remained after 30 min at 10 000 atm (16). These experiments, however, did not exclude the possibility of a reversible denaturation. In the same investigation it was also shown that the heat denaturation of DNA is counteracted by high pressure in spite of the fact that single stranded DNA has higher density than double stranded DNA. The authors concluded that this might show that the denaturation passed through a transient state with larger volume than that of double stranded DNA. The stabilizing effect of pressure against heat induced separation of the DNA chains has already been confirmed in other investigations using a different technique (17). It was finally shown that no denaturation, reversible or irreversible, occurred on the exposure of DNA to 9 000

atm at 30° C during 60 min (18). In that experiment formaldehyde was used to prevent a recombination of the strands if a reversible separation would have appeared.

The reason for the high stability of DNA as compared to proteins might be that proteins are weak amphotere polyelectrolytes with many undissociated groups which dissociate when the pressure affects the dissociation equilibrium, while DNA lacks corresponding undissociated groups.

Pressure effects on cellular synthesis of macromolecules. A common method in the investigation of macromolecular synthesis is to follow the incorporation of radioactive aminoacids and nucleotide bases. This has been done in Escherichia coli with ¹⁴C-adenine, ¹⁴D-glycine and ¹⁴C-leucine (19). The synthesis of nucleic acid (DNA + RNA) proved to be stimulated by 270 atm as compared to 1 atm. However, at 680 atm the rate of synthesis was low. The protein synthesis was also stimulated by 270 atm. This activity returned to its original at 408 atm and higher pressure inhibited the synthesis of protein, at 680 atm the inhibition was complete within 20 min. This work also illustrates the necessity to regard the temperature effect in high pressure experiments. The mentioned data for protein synthesis concerns 37° C. At lower temperatures the activation of the protein synthesis at 270 atm was less marked and at temperatures below 26° C the synthesis was inhibited even at 270 atm.

In another investigation on the incorporation of radioactive metabolites in anaerobically growing E. coli, the synthesis of DNA, RNA and protein was followed during three hours at pressures from 1 to 900 atm (20). The synthesis of RNA was the most pressure tolerant process which was only slightly retarded at 578 atm. However, at 800 atm the RNA synthesis was strongly inhibited. The DNA-synthesis displayed evident inhibition at 385 atm and it was completely stopped by 694 atm after 80 min. This lag was supposed to depend on the possibility of the cell to finish a commenced replication, while no new replication could be started. At 946 atm the replication was immediatly stopped.

The protein synthesis was the most pressure sensitive process. 378 - 490 atm caused a heavy but reversible inhibition. At 900 atm the synthesis was completely and irreversibly inhibited.

In summary it could be stated that the synthesis of protein is the most sensitive process and the synthesis of RNA is the most resistant process. Protein synthesis is affected at pressures above 200 atm and RNA at pressures above 500 atm. Approximately 600 atm stop the protein synthesis while 700 - 1 000 atm are required to stop the RNA synthesis (19, 20, 21, 22, 23).

The synthesis of RNA and proteins has also been studied in human cells (HeLa) (19). The pressure range from onset to complete inhibition of the RNA synthesis was 140 - 700 atm. The corresponding figures for protein synthesis was 300 - 600 atm. Thus, the macromolecular synthesis in human cells seems to show approximately the same sensitivity to pressure as does that of microorganisms.

These experiments on macromolecular synthesis under high pressure were run only for short times. Of course there is no long term synthesis of nucleic acids at pressures where protein synthesis is inhibited. Anyhow, the results point to what may happen initially when an organism is exposed to a high pressure.

The various steps in the protein synthesis have been studied in detail. Synthesis of the inducible β -galactosidase in E. coli was studied by two authors. But however, they used different strains (22, 23). An induced culture which was exposed to 450 atm ceased to synthesize β -galactosidase after a few minutes (22). The enzyme was not affected by 1 800 atm when exposed in vitro, which means that no inactivation of the enzyme occurred. Inhibition of the synthesis can be expected at different levels: Induction, transcription, translation and so on. Also effects on mRNA, tRNA or polysomes may be expected. The steps in the protein synthesis was divided into two groups: reactions before and after the synthesis of mRNA. By pulse inducing of the cells a pool of mRNA was produced which was then able to yield a certain amount of enzyme. The cells were pressurized after the pulse induction and then 70% of the enzyme activity in the control was found after 625 atm treatment. After treatment with 1 320 atm there was

still 10% activity in comparison to the control. Thus, the β -galactosidase synthesis, that was completely inhibited by 450 atm, must have been inhibited in a step before the mRNA, e.g. the transcription or the induction.

In the other work (23) the synthesis of β -galactosidase was more sensitive, but there were differences in the experimental conditions. Pressures exceeding 265 atm inhibited the synthesis, at 535 atm it was complete. The mRNA synthesis (transcription) was studied by the addition of proflavine and borate to induced cells after the pressure incubation and subsequent determination of the enzyme activity. These compounds prevent any new synthesis of mRNA and the enzyme activity was therefore a measure of the previously produced mRNA. The experiment disclosed that mRNA synthesis continued at 670 atm, a pressure that was completely inhibitory to the total synthesis. The induction in this experiment had occurred at 1 atm so the conclusion was that transcription was not stopped by 670 atm. The pressure effect on induction was studied by measurement of the enzyme activity after induction under pressure. This reaction turned out to be the most pressure sensitive part of the complete synthesis. Inhibition started at 67 atm. Further experiments revealed that the penetration of the inducer (IPTG) continued at 670 atm and the author concluded that the complex between inducer and repressor might be the target for this pressure effect.

The later steps in the protein synthesis have also been studied in detail (24). The polyU-mediated binding of ^{14}C -phe-tRNA to ribosomes of E. coli was somewhat inhibited (20%) at 100 atm and completely but reversibly at 600 atm. The complex phe-tRNA-ribosome was quickly dissociated by high pressures (1 000 atm). Also the peptide production (phenylalanylphenyl-alanine) turned out to be sensitive to pressures exceeding 200 atm and completely inhibited by 800 atm.

The production of mRNA-ribosome complex was studied by measurement of the binding of ^{14}C -polyU to ribosomes in a cell-free E. coli-system (24). Also in this case the authors demonstrated that a reversible dissociation of the complex started at pressures above 100 atm.

Recent investigations (25, 26, 27, 28) in cell-free bacterial systems emphasizes the influence of pressure on the process of binding of aminoacid-tRNA to ribosomes and to RNA as the main reason for pressure inhibition of protein synthesis. Furthermore these results deny the instability of the polysomes during pressure treatment. The properties of the ribosomes are of utmost importance for the pressure resistance which has been demonstrated in hybrid systems with the relatively pressure resistant Pseudomonas fluorescens and the more pressure sensitive E. coli (28). Pseudomonas-ribosomes + S-100 supernatant from E. coli had a sensitivity to pressure corresponding to that of the pseudomonad while E. coli-ribosomes with S-100 supernatant from the pseudomonad had a sensitivity to pressure like that of E. coli.

In the experiments referred to above the common experience was that about 500 atm is the upper limit for protein synthesis. However, there are existing organisms, mainly bacteria, in nature that can live at 1 000 atm, which shows that the synthesis of macromolecules can adapt to higher pressures. Actually such an adaptation can occur very quickly, which has been shown in cultivations of E. coli and Vibrio marinus cultivated at pressures alternating between 1 and 544 atm (29).

Pressure effects on cell division. A culture of Escherichia coli is slowly killed by pressures above 500 atm but if the cells are cultivated at somewhat lower pressure elongated cells (filaments) are formed. They have the normal diameter but the length can be some tenths of a millimeter (30). These filaments decompose within a few hours after decompression and form normal cells again. The tendency to form filaments during pressure exposure has been shown for several genus. (31, 32, 33).

The RNA content of the biomass in a filamentous E. coli culture grown under pressure increased with increasing pressure up to 450 atm and it was 60% higher at 450 atm as compared to 1 atm. However, the DNA content decreased simultaneously and it was only 35% of the content at 1 atm. The protein content did not change. It was evident that the amount of DNA per cell

remained constant during the filament formation, which indicates that the DNA synthesis was blocked though the cells continued to grow and produce protein and RNA (30). The DNA synthesis starts with partial cleavage of the double helix into two single strands of DNA. If this process passes via a stage of larger volume then it will be inhibited by increased pressure. This seems probable since the separation of the two DNA strands by heat has been shown to be reversed by pressure (16, 17, 18). There is another possible explanation which is that enzymes involved in the DNA - replication might be inhibited by pressure.

The cell division of eucaryotic cells has also been shown to be sensitive to pressure. It is the chromosome separation processes that cannot stand high pressure and the suggested reason for this is the effect of pressure on the cytoplasmic sol-gel equilibrium. The cytoplasm contains a molecular system (sol) which can be polymerized into a three dimensional mesh of fibrills (gel). This formation is entotherm and has $\Delta V > 0$ which means that the reaction is enhanced by increased temperature but retarded by increased pressure. It constitutes a mechanism for conversion of chemical energy in the form of ATP into mechanical energy and it forms the basis of ameboid movement and cell cleavage (separation of chromosomes and elongation of the cell). This situation means that the strength of the gel is pressure dependent. At 70 atm the strength is only 75% of the strength at 1 atm and at 700 atm only 10% of the original gel strength remains. The pressure sensitivity of eucaryotic cells has mainly been studied on fertilized eggs from Arbacia punctulata. However, this sensitivity seems to be approximately the same for other egg cells that have been tested. 400 atm breaks down the mitosis apparatus in these eggs within a few minutes. 140 atm is enough to slow down the migration of chromosomes and 280 atm stops this migration completely. At pressures below 500 atm these effects were reversible but at higher pressures irreversible damage occurred to the cells (34, 35). Eggs of the nematode Ascaris megalocephalia were much more resistant. The cell division was not inhibited until 800 atm (36).

High pressure has been reported to increase the mutation frequency of bacteria. Serratia marino rubra had 3 times higher the mutation rate to

streptomycin resistance at 300 atm as compared to 1 atm (37). Other investigations on Escherichia coli at 3 000 atm did not reveal any increased mutation rate caused by pressure (38). Euglena gracilis which was exposed to 500 - 1 000 atm during a few minutes mutated with high frequency to chlorophyll less mutants (39). There are also reports showing the opposite effect. High pressure has been reported to counteract the mutagenic effects of UV light and nitrogen mustard if the mutagenic dose is low but increase the effect at higher doses. This was taken as evidence that these mutations involved volume increasing subreactions (40).

Pressure effects on the energy metabolism. One possibility to measure the metabolic activity of an aerobic organism is to measure the oxygen consumption. Measurement of oxygen consumption of Tetrahymena pyriformis revealed that 272 atm depressed the consumption rate reversibly to 86% of that of the control. At 544 atm the oxygen consumption was only half that of the control and the treatment caused irreversible damage (41). Corresponding results were obtained from Ulva lactuca. Also in Escherichia coli the oxygen consumption was retarded by increased pressure (42).

In anaerobic respiration nitrate is sometimes used as electron acceptor instead of oxygen. This nitrate reduction has been studied in 30 species of marine bacteria cultivated at elevated pressure (43). At 1 000 atm there was still a respiratory activity even if it was reduced as compared to the 1 atm activity. Not until the pressure was raised to 1 400 - 1 800 atm during 24 hours did an irreversible inactivation of the anaerobic respiration occur.

Oxygen consumption measurements in higher organisms show another pattern. The oxygen consumption increased initially when Platichthys flesus and Carcinus maenas were compressed to 100 atm. After a few hours the rate returned to the original one (44). In Japanese works which have been reviewed in ref. (42) a general stimulation of the oxygen consumption occurred in muscle, kidney and heart tissues when pressure was increased up to 480 atm. Not until pressures exceeding 970 atm did the oxygen

consumption in these tissues decrease. In another study with organs from a frog an increased oxygen consumption was found in liver, kidney and lung tissues (45).

The ultimate purpose of respiration is to furnish the organism with energy or ATP. In an investigation on human cell cultures it was shown that the ATP content in FL amnion cells increased with 100% within 15 min after compression to 680 atm at 2 - 35° C. After decompression the ATP-content returned to its original value if the temperature was 35° C. If the temperature was 2° C the high ATP concentration remained on decompression. The ADP concentration was constant throughout the experiments which excludes the possibility of an effect just on the ADP-phosphorylation. However, primary amnion cells did not respond in this way. In that case the pressure did not affect the ATP content (46).

The hydrolysis of starch has been studied in more than 100 bacterial species. The rate of hydrolysis was proportional to the growth rate. The ability of starch hydrolysis remained in most species up to 300 - 600 atm. Some species could hydrolyze starch at 1 200 atm (47). These investigations were performed on living cells and the rate was therefore not only a function of the specific enzyme activity. In experiments in vitro with pure amylase it was shown that the hydrolysis was activated by increased pressure which is in accordance with theoretical speculations which shows that $\Delta \bar{V}^x$ for the hydrolysis of starch is about -25 cm³ (48). Also hydrolysis of sucrose with invertase from yeast is a reaction that is activated by high pressure. As optimum the rate of hydrolysis was 58% higher at 476 atm than at 1 atm (49). The utilization of hydrocarbons by deepsea bacteria under in situ pressure (500 atm) has been reported (50).

The glucose consumption has been studied in marine pseudomonas. The end product of the glucose metabolism of Pseudomonas desmolyticum was mainly cell mass and carbon dioxide at 1 atm. At 500 atm high contents of formic acid and almost no carbon dioxide was found as end products. Simultaneously with the decreased efficiency of the glucose metabolism its intensity increased when pressure was raised (51). In another marine pseudomonas which

was obligately barophile, i.e. it had a growth optimum above 1 atm, it was found that the glucose consumption per cell decreased when the growth rate increased and reached its maximum at 350 atm. Still higher pressure resulted in depressed growth and increased glucose consumption. At optimal pressure (350 atm) the end product of the glucose metabolism was mainly carbon dioxide but at higher or lower pressures larger amounts of volatile acids and less carbon dioxide was formed (33, 52). It seems as if the reduced efficiency of the glucose metabolism at too high or too low pressures to some degree could be compensated for by increased glucose consumption.

Some dehydrogenases in the TCA cycle (in E. coli) have been studied. A 15 min exposure to 600 atm irreversibly depressed the activities to 52, 74 and 81% respectively for succinate dehydrogenase, malate dehydrogenase and formate dehydrogenase (53). TCA-enzymes from mitochondria of Allomyces macrogynus have also been studied. α -ketoglutarate-, succinate-, oxalosuccinate- and isocitrate dehydrogenase were all completely inactivated by 600 atm during 72 hours. The fungi itself did not survive this treatment (54).

The susceptibility of the photosynthesis to high pressure has been investigated in several algae. At pressures from 200 to 600 atm there was a lineary decreasing activity measured as oxygen production or carbon dioxide fixation. The latter was somewhat more sensitive than oxygen production (55). In another work with the green algae Ulva lobata and the red algae Porphyra perforata 1 200 atm was required to stop the photosynthesis. The pressure effect on the photosynthesis was similar irrespective of which wavelength that had been used (56).

The sensitivity of some organisms to high pressure. The discussion above on biological reactions offers some hint of what may be expected when whole organisms are subjected to high pressure. From a general point of view one can say that the more complex the organism is the more sensitive it is to pressure.

Spores and viruses can resist thousands of atmospheres and no mammals will probably stand more than about a few hundreds of atmospheres. However, there are exceptions.

Viruses The inactivation rates of T2- T4- and T5-phages have been measured, (57). 2 000 - 2 500 atm inactivated 90% of these phages during a 5 min exposure. The same work also reveals that lysogenic E. coli (λ) can be induced to lysis by exposure to 290 - 1 040 atm. In another report, 2 000 atm for 30 min was not enough to inactivate completely encephalomyelitis virus, herpes virus, yellow fever virus and rabies virus (58). However, if the pressure was increased to 3 000 - 7 000 atm during the same time a complete inactivation was achieved. Viruses with exceptionally high sensitivity are phages of Actinomycetes which were inactivated by 700 atm. Only 1 phage per 10 000 survived this pressure. Lysogenic Actinomycetes were also inducible by pressure (59).

Spores. Several sporulating bacterial species from Bacillus as well as Clostridium were able to sporulate at 400 atm. Nor did their germination respond to this pressure. One species (Bacillus abyssus) sporulated more efficiently at 400 atm than at 1 atm (60, 61).

Spores of Bacillus subtilis survived 17 000 atm during 45 min (62). It was shown during sterilization experiments on milk that a small fraction (0.05%) survived 10 200 atm during 30 min (63). These organisms turned out to belong to sporeforming species and they had probably survived as spores.

The reason for the high resistance of spores to high pressures is probably the same as the reason for their resistance to temperature. The protein is protected against denaturation by stabilization with DPA and calcium, which protects their reactive groups from effects by steric changes caused by pressure or temperature. However, pressure has a remarkable effect on the germination of spores. The first publication on this showed that Bacillus pumilis spores, which were treated with 600 atm, were inactivated according to a first order kinetics (64, 65). This was a remarkably high sensitivity and it was shown that the reason was that the spores germinated when pres-

surized and that it was the germinated spores that were inactivated by the pressure. The volume change of activation calculated on basis of some germination associated reactions, viz. loss of heat resistance, loss of refractility and change in stainability, were calculated to $-139 - -158 \text{ cm}^3/\text{mol}$ which are relatively large volume changes. The negative sign ($\Delta V^X < 0$) shows that the reaction is promoted by pressure.

In another work (66, 67) it was also shown that lower pressures, which did not kill the germinated spores, were able to initiate the germination of several Bacillus species. As always, the pressure effect was very temperature dependent. In the easiest inducible organism (B. cereus) 500 atm during 30 min initiated the germination of 99.9993% of the spores at optimum conditions. 250 atm during 30 min was enough to induce 99% germination in B. coagulans and B. subtilis spores and 99.99% germination in B. cereus spores. These spores were heat activated. Non-activated spores also responded to pressure but to a lesser extent. Interestingly, D-alanine which normally counteracts germination had the opposite effect at 250 atm. This was due to the ability of pressure to adjust the recemization equilibrium towards L-alanine which is a very general germination initiator. Pressure increased the potency of the common germination initiator L-amino acid and it was suggested that pressure, like some other germination factors (e.g. ribosides) increases the permeability of L-amino acids into the spore.

As in the case of heat inactivation of enzymes moderate pressure counteracts the heat inactivation of spores (68).

Inactivation of bacteria by pressure. The sensitivity of bacteria to pressure varies very much. Most terrestrial species are killed slowly by 500 atm, mainly because no growth is able to occur. Marine species are some what more tolerant to pressure (60). Efforts to sterilize milk (63) showed that vegetative cells were killed in an exponential way during 10 - 15 min at 5 000 - 8 000 atm. However, spores survived this treatment. Inactivation curves for E. coli (57) revealed that 529 atm had a slow

killing effect while 1 058 atm reduced the viable cells to about one per thousand within a few minutes. Also in these experiments there was a logarithmic rate of inactivation and it was noticed that the compression or decompression had a killing effect. In other experiments (69) it was shown that repeated compression - decompressions between 1 and 1 000 atm did not influence the bacteria. The differences between these experiments were that in the former work the cells were in their growing phase while in the latter work the cells were in a stationary phase, which might mean that a metabolic process was the target of the compression - decompression effect. Further evidence was found when bacteria which were harvested from the sea at 40 atm pressure and recompressed in the laboratory grew slower than bacteria growing in situ (70).

Very quick compression - decompressions with a frequency of 50/sec have a pronounced killing effect on some species at as low a pressure as 30 - 80 atm (71).

Several efforts have been made to use hydrostatic pressure as a disinfectant (63, 71, 72, 73). However, the high pressure resistance of spores does not make it easy unless the pressure is combined with other agents like sudden pressure changes (71) or chemicals (72).

Bacterial growth under pressure. Bacteria that are able to grow at pressures over 400 - 500 atm are called barophile. Those which cannot grow up to 300 - 400 atm are called barophobe. Some marine species are barophile but most bacteria, marine as well as terrestrial, are barophobe or barotolerant, which means that they can survive pressures up to 1 000 atm for a long time but they are not able to grow at higher pressures than 400 - 500 atm.

Most experiments on cultivation of bacteria at high pressure have been done without oxygen or with only the amount of oxygen that is dissolved in the medium at the start of the culture. However, recently a method for cultivation of aerobic species at controlled oxygen concentration at high hydrostatic pressure has been published (225). The culture is performed

within gas permeable bags surrounded by a large volume of compressed liquid, containing the oxygen.

Living bacteria have been isolated from the largest depths in the sea at pressures corresponding to 1 000 atm and many of them have been cultivated in laboratory at in situ pressure (74). The highest pressure at which bacterial reproduction has been reported is 1 400 atm (75). As a consequence of the antagonistic effects of temperature and moderate pressure bacteria have been able to grow at 104° C at a pressure of 1 000 atm (15).

Among 65 marine bacteria 35 species could grow at 400 atm but only 7 at 600. All species grew best at 1 atm (60). However it has been reported that there exist bacteria with optimal growth at superatmospheric pressure (obligate barophiles) (33, 43, 76). A review with many data on bacterial growth under pressure has been published by ZoBell (69).

Except the effect of temperature on the pressure tolerance of bacteria the medium composition influences also this tolerance. This fact means that the figures given on the pressure limit for an organism must always be considered as an approximation. Cultivations of Vibrio marinus showed that additions of Na^+ , K^+ and NH_4^+ increased the barotolerance. Also some anions had a similar effect (77). In other experiments with Streptococcus faecalis the barotolerance was considerably increased by the addition of Ca^{2+} and Mg^{2+} but not by the addition of many other ions. The maximal pressure for growth in a complex medium was increased from 550 to 700 atm by the addition of 50 mmol Ca^{2+} or Mg^{2+} per liter medium. The same organism could not grow at pressures exceeding 300 atm if the medium contained pyruvate as the sole carbon source (78, 79). The effect of the ion composition of the medium on the barotolerance offers yet another touch to the analogy with temperature effect on life processes. Adaptation to higher pressure resistance can also occur on the enzymatic level (80).

Recently Pope and Berger (81) have proposed that the ultimate limit for microbial growth under pressure is the sensitivity of the protein synthesis of the organism.

Eucaryotic microorganisms. The higher microorganisms with eucaryotic cells (fungi, algae and protozoa) are mostly more sensitive to pressure than the procaryotic cells (bacteria and blue-green algae). The eucaryotic cells are, for their reproduction, dependent on a mitos apparatus that separates the chromosomes. This mitos apparatus belongs to the most pressure sensitive part of the eucaryotic cell. The sol-gel equilibrium, which was discussed previously, controls the mitos apparatus and it is very sensitive to pressure. The strength of the polymer gel phase is reduced with 50% by 200 atm pressure (34). Amoeba use the sol-gel equilibrium for their amoeboid movement which is also very sensitive to pressure. An investigation on Amoeba proteus showed that at 150 atm some pinocytosis channels disappeared and at 225 all the channels disappeared within a few minutes (82). The pseudopods were also reduced by pressure since the gel phase turned into sol phase. The pseudopods started to grow thinner at 100 atm and at 350 atm the cells became quite round. When the compression to 400 atm was sudden the pseudopods broke and separated from the cell since the plasma gel of the pseudopods were weaker than that of the body (83). Also Euglena (84) and Tetrahymena (85) species grew spheric when the cells were pressurized.

Higher organisms. There are a large diversity of barotolerances amongst the higher organisms as well as amongst microorganisms. No one knows how high a pressure man or mammals can stand. The situation is somewhat complicated when it comes to organisms which acquire their oxygen from the gas phase since it is difficult to exclude effects of the gases at compression.

The highest pressure a man had been exposed to in a pressure chamber in 1971 was 72 atm (86), but nothing indicates that this hydrostatic pressure is injurious to man. Mice have been exposed to 125 atm of a helium-oxygen atmosphere (87, 88) and some marine mammals can live for short times at 400 m depth in the sea which generates about 40 atm pressure (89). However all these figures concern shorter exposures and pressure might well cause long term effects on the organism at lower values. The tendency of protein synthesis to respond to pressures above 100 - 200 atm indicates that such complicated organisms as mammals and man would be severely injured by longterm exposure to such a high pressure.

Effects of high gas pressure

Definitions. When a liquid in a vessel is compressed this generates a hydrostatic pressure in the liquid. Common units for pressure are pascals (pa), which is the unit of the SI-system, atmospheres (atm), pounds per square inch (psi), and millimeter mercury (mmHg). If the force is exerted by a compressed gas which presses the surface then there will also be a gas pressure in the vessel. The total pressure of the gas is numerically equal to the hydrostatic pressure. Of course, systems exist with gas pressure without a hydrostatic pressure but in most biological systems there is also a water phase prevalent and thus a hydrostatic pressure. In engineering the term gauge pressure is sometimes used. This is the pressure in excess of normal atmosphere pressure. In order to distinguish from this the sentence absolute pressure is often used for total pressure.

What will be said here about the gas laws is strictly speaking valid only for ideal gases but it can be considered approximately valid for conditions that will be concerned in this literature review. The partial pressure of a gaseous compound is the pressure that the gas would exert if it was the only gas component in the gas phase. The total pressure is equal to the sum of the partial pressures (Dalton's law). Furthermore, the fraction of the total pressure that is caused by a gas component is equal to the fraction of the total number of moles in the gas phase with which the gas contributes. In other words the partial pressure is proportional to the mole fraction of a gas component. According to Avogadro's principle one mole of a gas occupies the same volume irrespective of which gas is present. From this can be concluded that the partial pressure of the gas component is equal to the volume percentage of the gas multiplied with the total gas pressure.

Effects of pressure on the solubility of gases. According to Henry's law the solubility of a gas in a liquid is directly proportional to the partial pressure of the gas. Dalton once showed that ~~this~~ solubility of one gas component in a mixture is not dependent on the other components in the gas.

This is strictly correct only for ideal gases which do not react chemically with the solvent, but gases like hydrogen, oxygen, nitrogen, methane, and the noble gases are approximately ideal under normal conditions which makes the relation useful. Amongst the gases discussed in this review it is only carbon dioxide that deviates so much from this relation that the consequences are easily detected.

Experimental data reveal that Henry's law is valid at pressures up to at least 100 - 200 atm for solutions of methane, nitrogen, hydrogen and oxygen in water. The solubility curve for carbon dioxide in water bends down below the theoretical one when pressure is increased. The solubilities are also dependent on the temperature and salt content. Increasing these parameters will decrease the gas solubility considerably (90, 91).

Effects of high oxygen pressure on growth and viability of microorganisms.

Some reports on growth of bacteria under high air or oxygen pressure showed improved growth. A series of bacterial species were cultivated in nutrient broth in oxygen atmospheres (92). Three strains of Staphylococcus aureus and one strain of Proteus vulgaris grew better the higher the oxygen pressure that was used (up to 4 atm). Aerobacter aerogenes, Bacillus subtilis, Escherichia coli and another Staphylococcus aureus strain showed optimal growth at 2 - 3 atm of oxygen. A third group consisting of Pseudomonas aeruginosa and Corynebacterium diphtheriae grew better at 1 atm of oxygen than at 1 atm of air and finally Streptococcus pyogenes displayed a growth that was independent of oxygen pressures from 0.2 to 4 atm. When these organisms were grown on solid media almost all strains were slightly retarded by 1 atm of oxygen. Similar results were reported on Staph. aureus, E. coli and P. aeruginosa (93, 94).

The enhancing effect of oxygen reported above is probably to some extent attributed to the pressure effect on the gas transfer rate. Many microorganisms have saturation constants (K_s) for growth on oxygen in the range 10^{-2} to 10^{-3} atm oxygen partial pressure (45) and it would be surprising if the K_s -values for the organisms above were very much higher than these.

But when the organisms consume oxygen there will be a gradient of oxygen tension within the cultivation vessel and the actual dissolved oxygen may therefore be much lower than the concentration in equilibrium with the gas phase. Stationary cultures will promote this gradient. The fact that the same organisms grown on agar plates were much more susceptible to high oxygen pressure supports this idea.

Many investigations report on growth inhibition caused by oxygen. Growth of E. coli and Staph. aureus in puls-aerated cultures (96) at 5 - 65 atm of air (1 - 13 atm O_2) partly inhibited the growth. In this case the aeration could be considered to be efficient enough to prevent too large a gradient of oxygen concentration. Similar results on the same species have been reported (97). A large amount of data on the effect of oxygen on growth of microorganisms is gathered in a review by Gottlieb (98). To summarize these one can say that oxygen seems to be inhibitory to growth at relatively low pressures above the normal oxygen pressure in air and at 5 atm of oxygen growth will seldom occur. However, the nutritional and experimental conditions seem to be very important for the tolerance.

As for killing of microorganisms with high oxygen pressure a great variety of results exists. Fungi and bacteria which were inoculated on agar plates and incubated in 10 atm of oxygen were not able to form colonies. If the plates were then transferred into normal air the bacteria started to grow immediately, where as the fungi required some time to recover. At maximum, the fungi could survive a few weeks at 10 atm of oxygen and the bacteria somewhat longer (99). In a similar examination of 103 fungal species 72 species survived 10 atm of oxygen over a period of 7 days while 22 species survived 14 days (100). When some bacterial species were incubated on membrane filters in 1 atm of oxygen for 18 hours all species except Aerobacter aerogenes were unable to grow. The killing of the others varied from 1% to 100% (101). In another experiment 100 bacterial strains were exposed on agar plates to 3 atm of oxygen during 24 hours. The result varied from no effect on the number of viable cells to complete killing (102).

Bacteria seem to be somewhat more resistant to oxygen than fungi which are more resistant than higher organisms. This has been used to protect fungal cultures from infections of nematodes (103).

Adaptation to higher oxygen resistance has been reported (104, 105, 106, 107). The basis for this will be looked into later on in the discussion as to the nature of the oxygen toxicity. In nature extreme adaptation has been found in the gas gland tissue of deep sea fishes. This gland will produce oxygen pressure up to 200 atm but the sensitivity of the fish to oxygen pressure was similar to that of other animals, i.e. it died within a day at oxygen pressures above 1 atm (108).

The effect of high oxygen pressure has been shown to be dependent on hydrostatic pressure (109). Several bacterial species which grew normally in 35 mg dissolved oxygen per liter medium (corresponding to 5 atm of air) at 1 atm were killed if the hydrostatic pressure was raised to 100 atm. If the dissolved oxygen content was 7 mg O_2 /l (corresponding to 1 atm of air) the same hydrostatic pressure did not influence growth. Even as low a hydrostatic pressure as 4 atm exerted a weak but bactericidal effect on cultures with 35 mg O_2 /l. This is interesting since it means that air pressure may be more deleterious than pure oxygen pressure with the corresponding partial pressure of oxygen. Synergistic action of antibiotics and oxygen has also been reported in some (110, 111) but not all (92, 112) cases.

The toxicity of oxygen. High oxygen pressure is injurious to any living organism. The sensitivity varies but mostly partial pressures above one atmosphere is deleterious. The phenomenon has been known for a long time and many theories on the mechanism of oxygen toxicity have been presented but still no complete explanation has been offered.

A theory supported by many an experimental evidence is the theory of oxidation of sulfhydryl groups (SH-groups) in co-enzymes or enzymes (113). Enzymes which are dependent on free SH-groups are especially sensitive to high oxygen pressure in vitro. This group includes many TCA-cycle and cytochrome enzymes. Examples of sensitive enzymes are succinate dehydrogenase, glyceraldehyde-3P-dehydrogenase, and pyruvate dehydrogenase. Some

other enzymes may be completely insensitive to oxygen. An enzyme which has been inactivated in vitro can often be reactivated by treatment with glutathion (GSH), cystein and other reducing substances. High substrate concentrations are also protective, which intimates that there are sensitive sites on the active surface of the enzyme which are oxidized by oxygen. Furthermore, a pure enzyme is much more sensitive to oxygen than a cell homogenate.

These results have been confirmed in in vivo studies. In higher animals the injuries by oxygen are first revealed in the central nervous system (CNS) as convulsions and later on as pulmonary injuries. Convulsions begin within a few minutes at 5 atm oxygen pressure and within an hour this oxygen pressure kills most animals. The symptom on CNS disappears on decompression to normal atmosphere. If substrates to FAD depending enzymes or GSH or cystein are injected intreperitoneally into mice prior to the oxygen treatment they aquire markedly enhanced resistance to oxygen toxicity. Substrates to NAD-depending enzymes do not offer this protection (114, 115, 116). A corresponding protective effect has been shown by substrates in bacterial cultures (117).

However, there are objections to this theory. Efforts have been made to measure the oxidation of SH-groups in vivo during oxygen treatment. In Ehrlich ascites tumor cells no change of cellular dehydrogenase activity was found when the cells were exposed to 5 atm of oxygen. Furthermore it was shown that the cells were able to keep GSH at a protective concentration during the exposure. Artificial decrease of GSH during the exposure immediately resulted in decreased dehydrogenase activity. Thus, it is doubtful if high oxygen pressure does reduce the activity of the cellular enzymes (118). The same conclusions were drawn from an investigation where it was shown that no reduction of the number of SH-groups occurred in cell-free extracts of E. coli (119).

Another theory on oxygen toxicity concerns the production of too high amounts of hydrogen peroxide during oxygen exposure (120). Many results from experiments with microorganisms support this theory. Hydrogen peroxide is normally produced in the cell by cytochrome oxidase catalyzed reactions in the electron transport chain. This peroxide is extremely toxic and it is removed by the enzymes catalase and peroxidase.

E. coli and B. subtilis cultures which were exposed to 10 atm of oxygen during 18 hours and then inoculated onto a fresh medium started to grow synchronously. The resistance to high oxygen pressure in such a culture was greatest immediately after cell division. This time coincided with the time of highest activity of catalase in the culture. If the cells were kept in the oxygen rich atmosphere the catalase activity increased and adaptation occurred(121). A corresponding increased resistance to oxygen during the period of catalase synthesis has also been shown in yeast, in which the ability to synthesize catalase rapidly decreases after cell division. Presumably as a consequence of this, the yeast did not grow at as high an oxygen pressure as did the bacteria (122).

Production of free radicals by oxidative enzyme systems has been suggested to be a mechanism behind oxygen toxicity (96). Such radicals should doubtless be injurious to the cell. Experimental support for this idea has been given. When mice were exposed to high oxygen pressure there was an increase in free radicals, especially in liver tissue (123). The mutagenic effect of high oxygen pressure (124) might be a consequence of production of free radicals in water corresponding to the mutagenicity of radicals formed by ionizing radiation.

The idea of radicals as causative agents in oxygen toxicity has recently been given substantial support (105, 106, 107). It was shown that superoxide dismutase plays an important role in the protection of microorganisms against oxygen. This enzyme catalyzes the reaction $O_2^- + O_2^- + 2H^+ = H_2O_2 + O_2$ and it has been shown to be ubiquitously prevalent in aerobic microorganisms (125).

Oxygen induced the superoxide dismutase in Streptococcus faecalis, E. coli B, and Saccharomyces cerevisiae. The enzyme activity covaried with the oxygen resistance. For instance, E. coli B increased its superoxide dismutase activity 25-fold when the growth conditions were changed from anaerobic to 5 atm of oxygen. The induction was a rapid process, half the final enzyme activity was reached 90 minutes after the onset of induction. However, E. coli K12his⁻ produced increased contents of catalase and of peroxidase but was unaffected in respect to the superoxide dismutase

when the oxygen pressure was increased. This organism did not adapt to high oxygen pressure. E. coli B which did adapt and gain superoxide dismutase activity on oxygen exposure, this organism did not increase its catalase activity on this exposure. Finally, B. subtilis did not adapt to high oxygen pressure and did not gain superoxide dismutase activity during exposure. However, the catalase activity increased in the organism on oxygen exposure. These facts offer a convincing support to the theory that high oxygen pressures may interfere with the cell by producing radicals.

Therapeutic use of high oxygen pressure. The effects that high partial pressure of oxygen exert on microorganisms have been utilized in treatment of infections. This method is mainly useful in the treatment of infections by anaerobic organisms like Clostridium. When a patient inhales a gas with high oxygen pressure the oxygen tension in the tissues of the patient is raised. A repeated treatment consisting of exposure to 2 - 3 atm of pure oxygen during a few hours has been used without serious effects on the patient by the oxygen itself (126, 127). Higher oxygen pressure or longer exposure will cause injuries on CNS and lungs. Amongst infections that have been successfully treated can be mentioned gas gangrene (Cl. perfringens) (128) and Actinomyces infection (127). Infections by Cl. tetanus are not as easily treated with high oxygen pressure. Certainly the organism and the toxin production is inhibited but the tetanus toxin which has already been produced is relatively stable and continues to injure the patient. The α -toxin from Cl. perfringens has a short lifetime and therefore the oxygen therapy gives a quick response (129, 130, 131).

Oxygen at 3 atm has been shown to enhance the action of polymyxin B, which has been suggested as a method in treatment of Pseudomonas aeruginosa infections (132). Common antibiotics like penicillin and tetracyclin can enhance the action of high oxygen pressure on Staphylococcus aureus (133).

Plasmodium-infections in rats have been successfully treated by 3 atm air pressure (134). Hyperbaric oxygen therapy is also used in treatment of myocardial infarcts (126) and in treatment of tumors in combination with radiotherapy (135).

The earlier mentioned synergism between oxygen toxicity and hydrostatic pressure (109) may offer other possibilities to increase the efficiency of the hyperbaric oxygen therapy, since inert gases might be used to cause a hydrostatic pressure. However the biological effect of these gases must then be taken into consideration.

Physiological effects of metabolically inert gases at increased pressure.

The noble gases helium, neon, argon, krypton and xenon have often been considered as biologically inert. In this discussion hydrogen, nitrogen, nitrous oxide, carbon dioxide and some hydrocarbons will also be included.

The noble gases are metabolically inert and the other gases, except carbon dioxide, are in most biological systems also metabolically inert. In this discussion these gases will be referred to as "inert gases". However, these gases exert biological effects which are caused by their physical rather than chemical properties. Certainly the reason for the relatively limited knowledge about these biological effects is the fact that super-atmospheric partial pressure is needed for demonstration of these effects of most of the gases.

Divers have long been aware about the dangers which shortly have been called diving sickness, which is actually a complex of several malfunctions with different ethiology: squeezes caused by compression of air cavities, oxygen poisoning, nitrogen narcosis and decompression illness by expanding air in cavities or bubble formation in tissues (bends) (136). Nitrogen acts as an anaesthetic on man at partial pressures exceeding 7 - 10 atm (89). These were the problems that started the research on the effects of other inert gases on man, and interest early on was focused on the noble gases.

Helium is now the most frequent dilutant in diver's gas. Experiments on mice show that they do not show any signs of narcosis from helium or neon with pressures up to 125 atm (87, 88). When the gas pressure was increased to 135 atm the animals died without any sign of narcosis irrespective of which of the two gases that was used (88). This makes it probable that it was the hydrostatic pressure that killed the animals. However there are

observations that show that helium can cause metabolic changes. Increased metabolic activity of mice and insects has been observed when the nitrogen was substituted by helium in the breathing gas (137, 138). In the case of higher animals such a response might be explained by the much higher heat conductivity of helium when compared with nitrogen but when the same experience was found in Drosophila no compensation for losses of heat is likely (137). Specific changes in the metabolism of mice have been shown when exposed to helium atmospheres at high pressure. There was a direct relationship between the helium pressure and the content of γ -amino butyric acid (GABA) in the brain at 1 - 60 atm helium. As minor changes as substitution of the 0.8 atm of nitrogen in air with 0.8 atm of helium caused an increase in the GABA-content of the brain (139). Changes of enzyme activities in the serum (140) and liver (138, 226) of mice were also noticed at high helium pressure (1 - 60 atm). In man the exchange of the nitrogen of air with helium causes changed electrolyte balance, at compression to 10 atm (141). A similar electrolyte imbalance has been reported in cats under inert gas narcosis (142). In short one can say that helium may cause effects on the metabolism but that it has not proved to be able to cause narcosis.

The anaesthetic effect of the other gases increases with increasing partial pressure and approximately with increasing molecular weight of the gas. For most of the gases a superatmospheric pressure is needed to cause narcosis. The pressure limit for anaesthesia by the gas is somewhat dependent on the organism and on the definition of when anaesthesia starts. The following values of anaesthetic pressure are collected in ref 143:

N_2 29 atm, Ar 20 atm, CH_4 4.6 atm, Kr 2.9 atm, C_2H_6 1.3 atm, N_2O 0.90 atm, Xe 0.85 atm, and CO_2 0.25 atm.

Experiments with man and higher animals involves the possibility of influences of a psychological origin. In microbiological experiments and in experiments with biological systems in vitro these risks are eliminated. There are several microbiological experiments which confirm the results on higher organisms.

Hela cells which were cultivated in constant oxygen and carbon dioxide pressures with varying pressures of inert gases were inhibited by the inert gases. At 70 atm, helium and neon had a slightly inhibitory effect on growth (80 - 90% of the control). Nitrogen at 70 atm retarded growth to 60 - 70% of the control. Argon caused the same inhibition at 35 atm and krypton, xenon and nitrous oxide caused 50% inhibition at approximately 18, 4 and 4 atm respectively (144).

Corresponding results are reported with Neurospora crassa cultivated at 1 atm total pressure with constant pressure of carbon dioxide, oxygen and water vapor and with 0.91 atm of inert gas. The linear growth rate of the hyphae was directly proportional to the square root of the molecular weight of helium, neon, nitrogen, argon, krypton and xenon. The latter gas reduced the growth rate to about 60% of that of the control (nitrogen) and helium permitted a growth rate that was about 20% higher than that of the control (145). The same author has reported that this krypton atmosphere retarded the sporulation and stopped the cytoplasmic streaming within the hyphae. The xenon atmosphere inhibited completely both sporulation and cytoplasmic streaming (146).

In another experiment with Chlorella sorokiniana in 2.9 atm of helium, neon, argon and nitrogen at constant carbon dioxide pressure no significant differences in growth rate were found (147). In measurement of β -galactoside uptake in E. coli at 68 atm of He it was shown that this atmosphere increased the permeation by an effect on the β -galactoside permease. This effect was probably not due to the hydrostatic pressure since it was not temperature specific within 15- 37^o C (148). The same atmosphere also shortened the lag phase in E. coli by eliciting a more rapid iron transport into the cell (149).

The experiments on microorganisms related above involves only aerobic metabolism. The ideal system for studies of the inert gas effects on metabolism is anaerobic microorganisms in which effects on the oxygen transfer are excluded. Streptococcus faecalis was cultivated anaerobically at 1 and 41 atm of nitrogen, argon and helium and at 1 to 8 atm of nitrous oxide and xenon. It was also possible to use hydrostatic pressure in a gas

free system as a control. The growth inhibitory potential of these agents were $N_2O = Xe > Ar > N_2 > \text{hydrostatic pressure} > He$. Helium promoted the growth rate with about 7% when compared with hydrostatic pressure (150).

It seems obvious that these gases can cause narcosis in man and animals and that they can inhibit growth of microorganisms and human cells. Several theories have been offered concerning this inert gas effect.

One theory suggests that the inert gases compete with oxygen for space on specific sites in the cell. This does not seem to explain the results on Streptococcus faecalis referred to above, but there are many other experiments that support this theory. Oxygen promotes the mutagenic effect of radiation. This oxygen dependent radio sensitivity was reduced by inert gases when Vicia faba was irradiated with X-rays. The protective effect was correlated with the anaesthetic effect of these gases. 50 percent reduction of the oxygen dependent radiosensitivity in this organism was achieved by 55 atm of hydrogen, 55 atm of helium, 12,5 atm of nitrogen, 2 atm of argon, 2 atm of krypton or 1.1 atm of xenon. When Ehrlich ascites tumor cells were irradiated, 110 atm of hydrogen or nitrogen reduced the oxygen dependent radio sensitivity to 54% and 38% respectively. 21 atm of argon completely extinguished the oxygen effect. Also in bacteria (Shigella flexneri) argon and nitrogen reduced the oxygen dependent radio sensitivity. Since the relative potency of the gases were so similar to their anaesthetic power the authors also tested cyclopropane which is a well known anaestheticum. 2 atm of cyclopropane reduced 50 percent of the oxygen dependent radio sensitivity in Vicia faba. The authors suggested that the site of displacement of oxygen by the inert gases was the interphase between lipid and water or perhaps in a lipid fraction of the cell (151).

Besides these in vivo experiments there is evidence on the competition for space between oxygen and inert gases also from in vitro experiments with enzymes. The oxygen dependent enzyme tyrosinase was inhibited by 42 atm of nitrous oxide or 350 atm of nitrogen or argon in a gel but not in liquid. The oxygen independent enzymes invertase, trypsin and chymotrypsin were not inhibited by the same treatment (152). Tyrosinase was inhibited by several inert gases at 30 atm in other experiments but in that report the oxygen independent acetylcholine esterase was also inhibited in a similar way (153).

The striking similarity of the effects of inert gases and traditional anaesthetics have suggested an effect of the gas on membranes. The common anaesthetics as well as the inert gases have a solubility in lipids which is very well correlated to their efficiency as anaesthetics (154). If the gases dissolve in membranes they might very well influence their properties. There are several suggestions as to how the gases influence the membrane.

In experiments with oil emulsions exposed to high inert gas pressure and anaesthetics, it was shown that there was a tendency of the inert gases and anaesthetics to reverse oil-in-water emulsion to water-in-oil emulsion. Such a tendency should certainly reduce the permeability for water dissolved molecules or ions through the emulsion (155, 156).

In experiments where the surface tension of phospholipids was measured under inert gas pressure it was shown that carbon dioxide, oxygen, argon and nitrogen in falling order of potency decreased the surface tension and that helium and neon did not affect this tension (157). In another experiment (158) 0.6 atm of nitrous oxide which is an anaesthetic for man, decreased the surface tension about 0.4 dyn/cm in a lipoprotein monolayer. Such a change in surface tension was caused by 6 atm of nitrogen and by 3.8 atm of argon in the former experiment (157). These pressures are fairly well correlated to the anaesthetic pressures of these gases. As high a pressure as 112 atm of helium did not cause this degree of decrease of surface tension (157) and helium at this pressure is not an anaesthetic (88).

According to these investigations a decrease of permeability of water solutions through membranes ought to be expected on the exposure to inert gases. However measurements in vivo shows that anaesthetics and inert gases at anaesthetic pressures cause a reversible increase of cation permeability through membranes. This increase of permeability is related to the anaesthetic potency (159, 142, 141).

Still another type of interaction between the inert gases and the cell has been suggested in two rather similar and simultaneously published theories

(160, 143). They suggest the formation of gas-water complex within the cell. Hydrates of some of the inert gases are known, but they have not been found during natural temperature. However, it is suggested in one theory (143) that the gas molecules become surrounded by ice-like configurations of water("icebergs"). According to the other theory (160) true gashydrates are formed in the cell during narcosis because they are stabilized by side chains of proteins. Both theories emphasize the binding of water to clusters which spatially interferes with, above all, membranes and change their permeability properties. The support to both theories is mainly gained from the fact that there is a good correlation between the anaesthetic pressure of the gas and the dissociation pressure of the gashydrate.

Recent investigations rather focus the interest on the lipid solubility theories (161, 162, 163). These investigations are concerned with the antagonistic effect of hydrostatic pressure against anaesthesia.

It has been shown in newts (Triturus cristatus carnifex), mice (161, 162), Tetrahymena pyriformis (162) and in vitro on the permeability of liposomes to ions (162), that the effects of anaesthetics can be reversed by hydrostatic pressure. The pressure that was needed to reverse the smallest anaesthetic inhibitory effects was in the order of 150 atm. The higher the concentration of anaesthetics, the higher the pressure was needed. This phenomenon has been treated theoretically on the basis of the partial molar volume and the lipid solubility of the anaesthetic, which tend to increase the volume of the site of action in the cell, and the volume decreasing effect of hydrostatic pressure (161). It was calculated that an anaesthetic dose would cause an expansion of lipid in the order of 0.4% and that this expansion would be reversed by 100 atm hydrostatic pressure. This is consistent with the experiments mentioned above (161, 162, 163). Two sites of action of the anaesthetic was suggested (161): hydrophobic areas within an enzyme and/or the lipids of membranes.

There is still no direct proof for the mechanism of inert gas effects on biological systems. Good correlations between the effects, whether measured as anaesthesia or growth inhibition, have been found with molecular weight

(145), solubility in oil (154), molecular polarizability (153), gas hydrate dissociation pressure (160, 143), effects on surface tension of lipids (157, 148) and effects on oxygen action (151).

The most delicate effect of these gases is probably their ability to depress the mental activities of man, but they also cause a type of general inhibition of life processes in vivo, an inhibition which seems to be reversible and not injurious to the organism if the treatment has not been too hard. The results of this thesis will include the ability to inhibit the germination process in bacterial spores, a process which certainly is a very primitive life process.

EQUIPMENT FOR CULTIVATION OF METHANE OXIDIZING BACTERIA AT ELEVATED PRESSURE OF EXPLOSIVE ATMOSPHERES

Summary

An equipment for small batch wise cultivations of bacteria under high pressure of explosive gases is described. The equipment was designed for studies on the effects of elevated pressure of methane and oxygen on growth of methane oxidizing bacteria. The growth is measured automatically at intervals by means of a photometer which is partly built into the pressure vessel.

Introduction

When bacteria are to be studied at high pressure of gases that are or may become explosive, some very special problems arise. The high pressure per se offers problems in the analysis and control of the process and the explosive atmosphere requires special attention to the design of the equipment. Two principles can be used for the design:

1. No possibility of ignition of the gas.
2. The amount of explosive gas is kept so small that an accidental ignition will be safe.

The former principle is the only one that can be used in larger scale cultivation. However, it is hazardous to use a flammable mixture of gas and hope that there is no ignition source since friction heat in bearings and electrostatic discharges are ignition sources which are very difficult to control. If, however, the composition of the gas is controlled there are some possibilities to increase the safety. A gas mixture like methane in air at 1 atm is flammable only when the concentration of methane is between 5 and 15%. The corresponding figures for hydrogen in air are 4 and 75% respectively. It must be noticed, however, that these figures are very rough and largely depending on experimental conditions (164, 165, 166).

The safety can be improved by dilution of the mixture with an inert gas like nitrogen or carbon dioxide. Thus, if a methane-air mixture is diluted so that the oxygen content is below 12%, this mixture will not be explosive at atmospheric pressure whatever the methane content might be (166).

Double safety can thus be achieved by using a gas mixture with less than 12% of oxygen and less than 5% of methane. However, this atmosphere will give a very bad gas transfer rate. Furthermore, the use of an extra gas for dilution in a large plant will increase the costs to an unrealistic level, unless a gas recirculation system is used.

A special solution to this problem has been suggested (167). This method uses separate emulsification of air and methane in the medium before they are mixed together in the fermenter. The gas emulsion is then promoted in the fermenter by a surface active agent so that no large continuous gas phase will appear until after the exit from the fermenter through a foam separator.

The second principle mentioned above offers some advantages in small scale experiments since it will not restrict in the choice of gas mixture. The major disadvantage in this case is the limited volumes of gas and culture that must be used. As a guide in the estimation of the pressure that can arise if a small gas mixture is ignited within a pressure vessel, a work by H. Busch can be used (168). For instance, if 5 l of a methane in air mixture explodes in a 5 l pressure vessel this will generate a maximum pressure of 6 atm. This pressure will be reached if 9% methane is used.

The purpose of this work was to construct an equipment which made it possible to study the effects of high methane and oxygen pressures on the growth of methane oxidizing bacteria. Since this task demanded freedom in the choice of gas mixture the equipment was designed according to the principle that the amount of gas should be so small that the pressure vessel would withstand an accidental ignition. Measurement of growth under elevated pressure was considered to be the most important aim of this work. Therefore the apparatus was furnished with equipment for automatic recording of optical density of the culture. This made it possible to make frequent measurements without sampling from the culture which would have been a problem with respect to the necessity to keep the culture volume small.

The cultivation equipment

The cultivation vessel with analytical equipment and pressure vessel is shown in fig. 1. The cultivation vessel consists of a stainless steel

bottle with a glass bottom. The total volume of the vessel is 60 ml and a culture volume of 25 ml has been used. It is attached to a shaking machine which consists of a pneumatic double action cylinder. This cylinder is fixed to the inside of the lid of a lying thermostated (jacketed) pressure vessel with the inner diameter 148 mm and the depth 280 mm (volume approximately 4.8 l). The pressure vessel was available in the laboratory and designed for 150 atm, which of course is a higher degree of safety than is necessary. The pneumatic cylinder is operated by alternating air pulses which are generated from outside the pressure vessel. The cultivation vessel is furnished with gas from a double walled rubber balloon (one balloon inside the other one) with 500 ml of the cultivation gas. The connection between this reservoir and the cultivation vessel consists of a 2 mm diameter stainless steel tube which is made flexible by winding it into a helix. In order to increase the pressure over the culture the pressure vessel is filled with nitrogen to the desired pressure level. Furthermore the use of nitrogen instead of air for this compression reduces the explosion risk. The gas mixture in the reservoir will be compressed simultaneously with the nitrogen compression and partly transferred into the cultivation vessel via the helical tube. At pressure equilibrium the total gas pressure inside the cultivation vessel will be the same as the nitrogen pressure in the pressure vessel. Since there are no differences in total pressure over the cultivation vessel walls, there are no requirements to choose pressure resistant materials for constructions.

The first experiments with this equipment revealed insufficient mixing between the gas phases in the reservoir and the cultivation vessel. The nitrogen pressure is therefore allowed to pulsate each 30 min. so that it increases 2 atm and immediately after that decreases again to the original pressure. This operation causes some of the gas to pass from the reservoir to the cultivation vessel, mix with the other part of the gas, and then return to the reservoir. The very short time for this operation is not likely to allow the increased concentration of dissolved gases to establish an influence on the growth parameters that are measured. The efficiency of this mixing procedure depends on the pressure at which it starts. Fig. 2 shows graphically how the reservoir volume varies with the total pressure.

The amount of gas included in this equipment is enough to furnish a culture of methane oxidizing bacteria of low cell density. At an increase in cell density of 0.3 g/l and a yield coefficient for methane of 1.0, the expected methane consumption will be 7 mg methane per culture. This corresponds to 2% of the total amount of gas. It must be noticed, however, that the partial pressures cannot be kept constant throughout the cultivation because of the gas consumption. Nor can the disappearance of gas be used for gas consumption calculations since an appreciable diffusion will occur through the rubber walls.

Measurement of growth

Measurement of reflected light was chosen as the basis for measurement of growth. A small lamp (12 V/16 W) is placed below the culture vessel in one of its extreme positions. The light beam passes an iris aperture and a glass filter (610 nm). The light then reaches the culture where part of it is reflected and measured by means of a photo resistor. An additional photo resistor is placed below the lamp and adjusted to compensate for changes in light intensity. The remaining electronics for the light measurement is placed outside the pressure vessel. Electrical signals between these parts of the instrument are transferred through a special device which is shown in fig 1. Since the measurement of reflected light is impossible to record as long as the culture is shaken, a controller is used to stop the shaking apparatus, switch on the photometer, and record the signal at intervals of 30 minutes. This gives a semicontinuous record of the growth curve (see fig 3). The μA -signal of the photometer is translated into absorbancy units (OD^{620}) which have been used in all calculations. The relation between OD^{620} -units and the instrument signal in μA is shown in fig. 4. The relation between OD units and dry weights is not constant over a broad range but Lambert-Beers' law is approximately valid for the concentration interval that is relevant in this investigation ($\text{OD}^{620} = 0.01 - 0.40$) (fig. 4).

According to definitions the specific growth rate is given by $N = N_0 \cdot e^{\mu t}$, where N_0 and N are the number of organisms at zero time and after "t" hours respectively. Derivation and rearrangement gives the formula $\mu = \frac{d(\ln N)}{dt}$. According to fig. 4 there is a relation between the optical density and number of cells per unit volume (N/V) that can be written $N/V = \text{const.} \cdot \text{OD}$. This makes it possible to calculate the specific growth

rate from $\mu = \frac{d(\ln OD)}{dt}$, or written on $^{10}\log$ basis: $\mu = \frac{d(\log OD)}{dt} \cdot \frac{1}{\log e}$. This formula was used to calculate the growth rate from the growth curves which had been rewritten on OD-basis.

The pressure limit for this equipment has not been established. The most critical point is probably the resistance of the lamp bulb. It was found by experience that repeated compression-decompressions to 10 atm offered no problems. A possibility to overcome the problems with bulb fractionations at higher pressure is to introduce optical fibres into the pressure vessel. This would also eliminate the most probable source for ignition of the gas mixture. The gas reservoir is another limiting part. The higher the total pressure the larger must the gas reservoir be in relation to the gas volume of the cultivation vessel to avoid a negative pressure on the cultivation vessel.

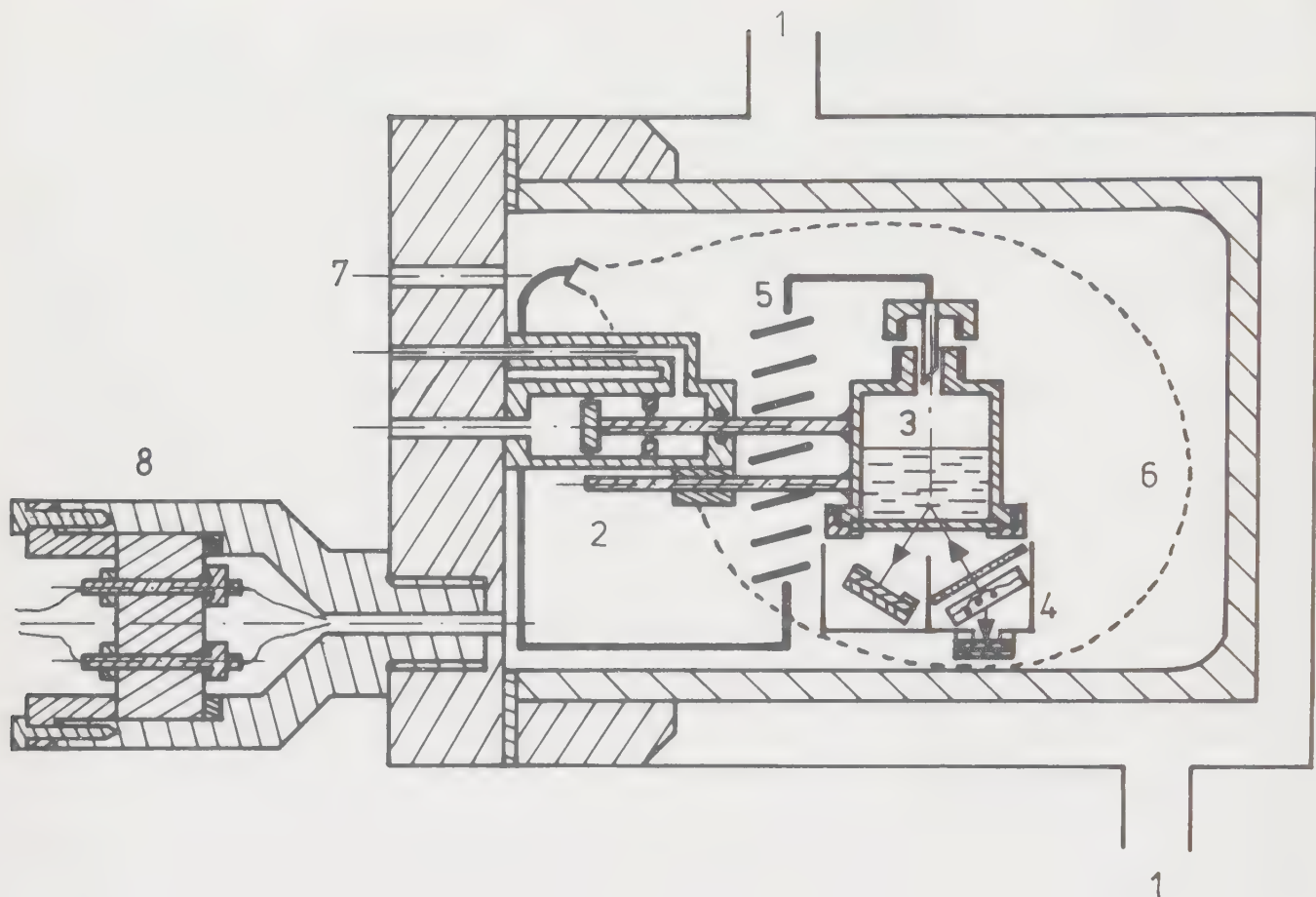


FIG 1. EQUIPMENT FOR MEASUREMENT OF GROWTH RATE OF BACTERIA IN EXPLOSIVE ATMOSPHERES.

1. JACKET FOR THERMOSTATING WATER
2. SHAKER
3. CULTIVATION VESSEL
4. PHOTOMETER
5. CONNECTION BETWEEN GAS RESERVOIR AND CULTIVATION VESSEL
6. GAS RESERVOIR (BEHIND THE CULTIVATION VESSEL)
7. INLET FOR NITROGEN GAS
8. ELECTRICAL CONNECTORS

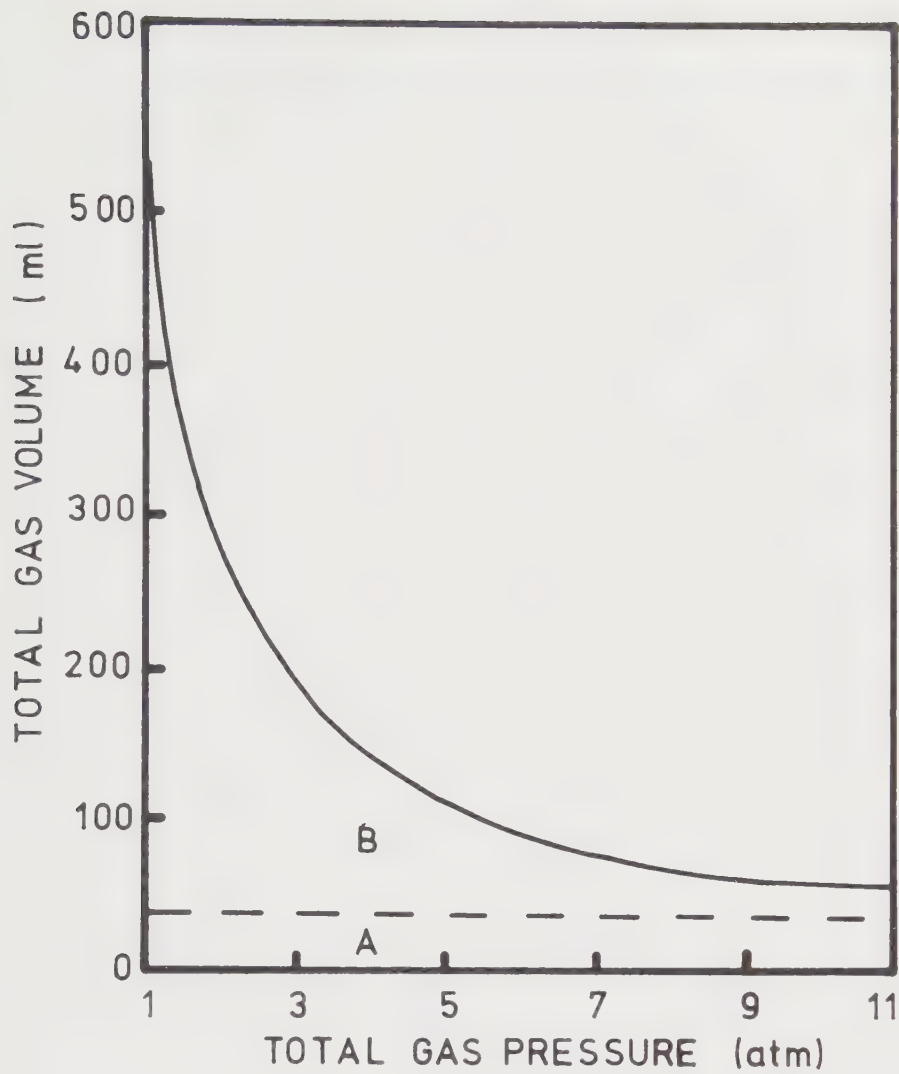


FIG 2. THE VOLUME OF THE GAS RESERVOIR (B) AS COMPARED TO THE VOLUME OF GAS IN THE CULTIVATION VESSEL (A) AT INCREASED TOTAL GAS PRESSURE.

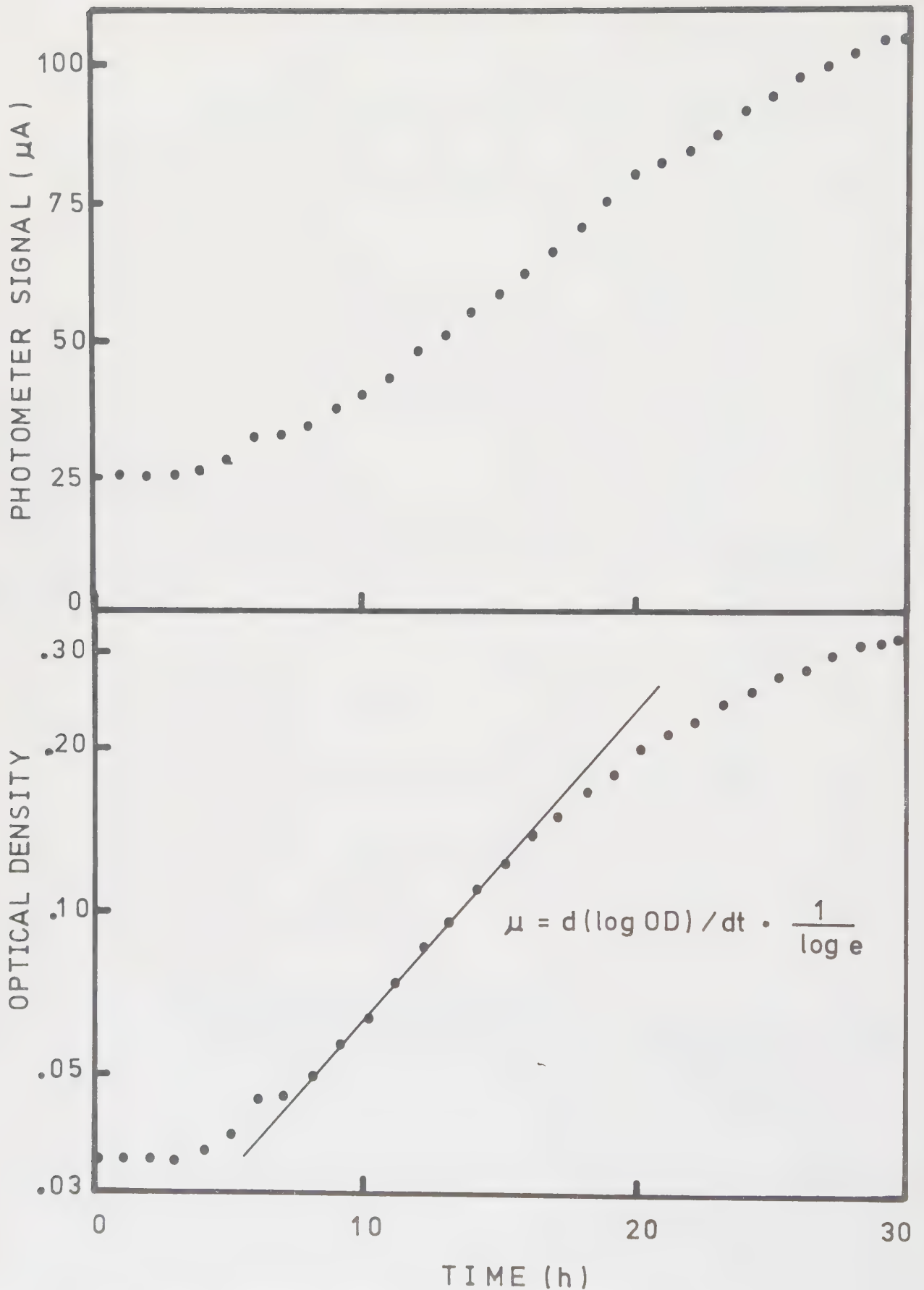


FIG 3. GROWTH CURVE AS RECORDED BY THE EQUIPMENT DESCRIBED IN TEXT (UPPER CURVE). THE LOWER CURVE SHOWS THIS CURVE TRANSLATED INTO OD-UNITS BY THE RELATION IN FIG 4.

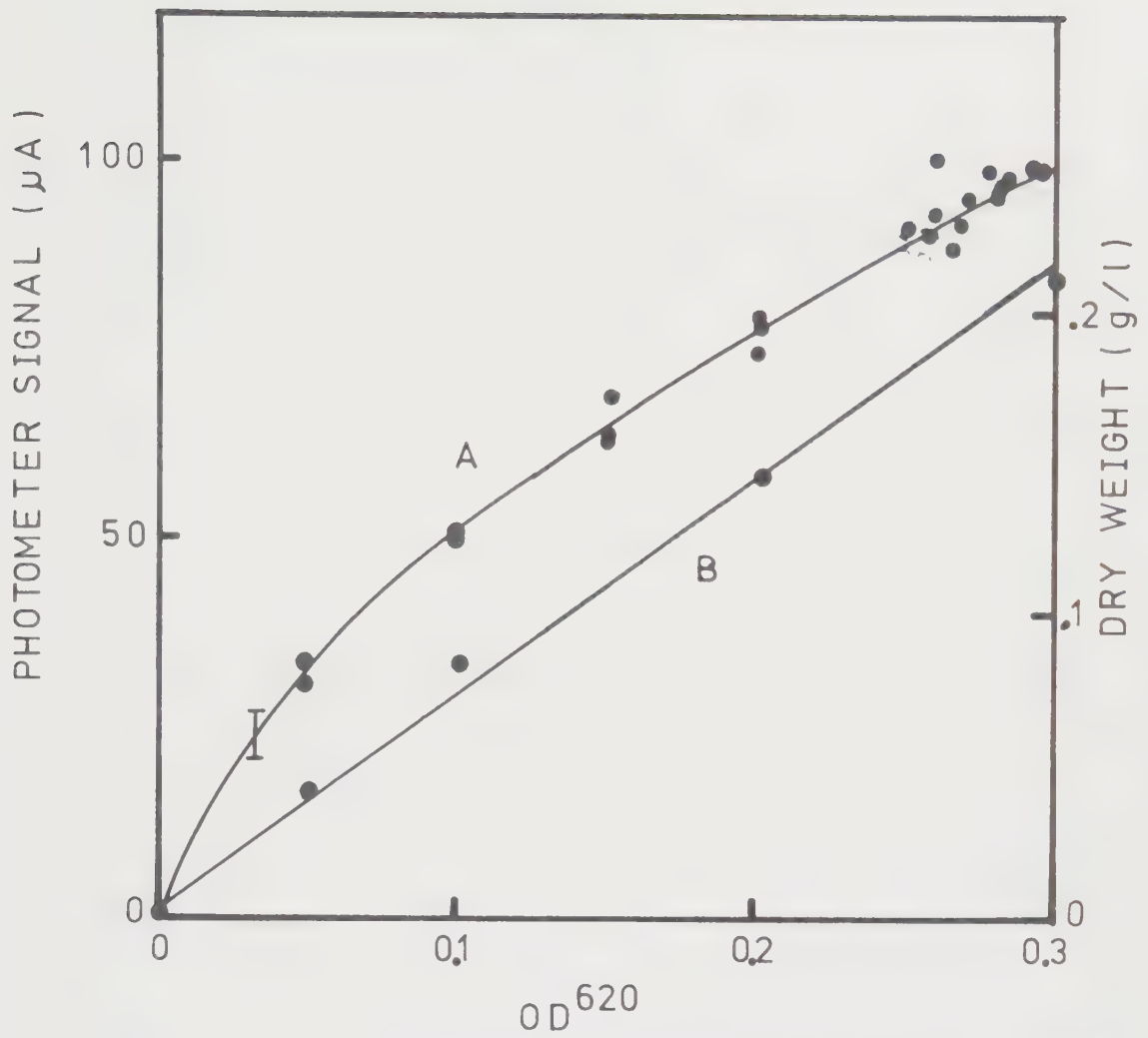


FIG 4. RELATIONSHIPS BETWEEN THE SIGNAL FROM THE BUILT IN PHOTOMETER AND OPTICAL DENSITY OF THE BACTERIAL CULTURE TM 10 (CURVE A) AND BETWEEN THE DRY WEITHTS OF THIS CULTURE AND THE OPTICAL DENSITY (CURVE B).

CULTIVATION OF METHANE OXIDIZING BACTERIA AT INCREASED GAS PRESSURE

Summary

Effects of the partial pressures of methane and oxygen on the growth of a mixed culture of methane oxidizing bacteria have been investigated. Increased methane pressure up to about 1 atm improved the growth rate. K_s for methane was calculated to 0.08 atm and μ_{max} to 0.153 h^{-1} . 2.8 atm of oxygen completely inhibited the growth and injured the culture. 5.5 atm of methane improved the growth, as compared to 0.5 atm of methane, in enrichment cultures and on solid medium but did not offer any advantages in the isolation of pure strains. One strain of methane oxidizing bacteria was isolated on silica gel but its capacity to grow in liquid cultures and on silica gel gradually ceased. Comparisons of agar, agarose and silica gel as a basis for isolation experiments were done. Abundant growth of non-methane oxidizers on agar plates turned out to be a problem.

Introduction

Methane oxidizing bacteria are sources of single cell protein which are of interest mainly because of the cheap raw material for their cultivation. They can be grown in an aerated water solution of some mineral salts with ammonia or nitrate as nitrogen source and with methane as carbon and energy source (169, 170). Methane is one of the cheapest available carbon sources for production of biomass. Another advantage with methane as raw material for biomass production (compared with the use of other hydrocarbons) is that it is non-toxic and that there is no problem in separating the cells from this hydrocarbon (171, 172).

However, combined with the use of methane some severe problems exist. Under certain conditions methane-air mixtures are explosive, (165) and the growth rate and productivity of methane oxidizing bacteria are relatively low compared with data from biomass production on methanol and higher hydrocarbons. Furthermore the oxygen requirement is critically high (173, 174, 175).

There could be set up many hypothesis why the methane oxidizing bacteria grow so slowly. One possibility is that too few strains have been examined as yet and that better suitable strains exist. Recent publications show examples of relatively fast growing strains. The knowledge about these bacteria has been restricted until recently when Whittenbury and co-workers published a series of papers on their extensive work with methane oxidizing bacteria (176, 177, 178, 179). This work shows that these bacteria constitute a more diverse group than was earlier known. Their common feature is that they can all grow with methane as the sole carbon source, and that they are furnished with membrane systems very similar to those in nitrogen fixing bacteria. These membranes are thought to play a role in the methane oxidation process (179).

The organisms can be classified in two groups (I and II) according to the morphology of the membranes. Whittenbury could correlate the type of membrane to the growth rate of the organism. Type II membrane (Methylosinus and Methylococcus) have typically 5 - 6 hours generation time whilst the other genera (Methylocystis, Methylomonas and Methylobacter) with type I membrane grew with 3 - 5 hours doubling time. The two different membranes have been correlated with different metabolic pathways (180, 181). The literature on methane oxidizing bacteria has been reviewed recently (182).

Another possible reason that the organisms used for SCP production from methane grow so slowly may be that the concentration of the carbon source (methane) is too low in many cultures: It is only the dissolved methane that is likely to be used by the bacteria. The solubility of methane in water at 30°C and 1 atm partial pressure is only about 15 mg per liter. Dissolving salts in the water or increasing the temperature will decrease this solubility still more (91). In a mixture of methane and air at atmospheric pressure, the partial pressure of methane will be lower than 1 atm. Most works reported on methane oxidizers have been done in an atmosphere with about 50% methane in air (183). That means that the initial amount of dissolved methane have mostly been not more than about 5 - 10 mg/l. The concentration of carbohydrate which is needed to permit half maximum growth rate of microorganisms, the saturation constant, is often in the order of 10 mg/l (184).

If this constant is of the same order for methane in methane oxidizing cultures, then it is obvious that low concentration of dissolved methane might be a reason for the generally slow growth rates in these cultures. The easiest way to increase the concentration of dissolved methane is to increase its partial pressure. This can be done under atmospheric pressure by increasing the relative amount (percentage) of methane in the gas phase. Methane oxidizing bacteria are, however, obligate aerobes, which means that a certain amount of oxygen must be prevalent in the mixture. That means that it is impossible to reach such a high concentration as 15 mg/l when working at atmospheric pressure. A possibility to increase the solubility of methane almost infinitely is to increase its partial pressure by increasing the total pressure of the gas mixture. The solubility of methane in water follows Henry's law up to at least 100 atm (91, 185).

The purpose of this study was to investigate whether the methane oxidizing culture TM10 which was used in a single cell protein production project (173) could be cultivated under elevated gas pressure and how the growth rate responded to increased partial pressures of methane and oxygen.

Materials and methods

Cultivation equipment. The equipment described in the previous chapter was used for recording the cell growth during cultivation.

Inoculum. A mixed culture of methane and methanol oxidizing bacteria (TM 10) which was isolated at the Department of Applied Microbiology, Karolinska Institutet, was used. The features of this culture is described elsewhere (173). The inoculum was taken from a turbidostat which was run at $OD^{620} = 0.5$. This culture was restarted each second week with lyophilized cells from one batch. The pressure cultivation apparatus was inoculated with $2.2 \cdot 10^8$ cells/ml which yielded the turbidity $OD^{620} = 0.035$.

Medium. $Na_2HPO_4 \cdot 2H_2O$ 0.6 g/l, KH_2PO_4 0.4 g/l, $MgSO_4 \cdot 7H_2O$ 0.2 g/l, NH_4Cl 0.8 g/l, $FeCl_3 \cdot 6H_2O$ 8.35 mg/l, $CaCl_2 \cdot 2H_2O$ 0.33 mg/l, $ZnSO_4 \cdot 7H_2O$ 0.09 mg/l, $CuSO_4 \cdot 5H_2O$ 0.08 mg/l, $MnSO_4 \cdot 4H_2O$ 0.08 mg/l, $CoCl_2 \cdot 6H_2O$ 0.09 mg/l. pH was 6.8 at the beginning of the cultivation but decreased as growth proceeded. To this medium was added for agar plates

15 g/l agarose (BDH electrophoresis grade) and for silica gel plates 240 ml/l Ludox (Du Pont). The methane used was Matheson Technical grade.

Temperature. All experiments have been performed at $30 \pm 1^{\circ}\text{C}$.

Measurement of growth. The cell density measuring instrument in the pressure cultivation apparatus measured and recorded each half hour the amount of light reflected against the culture. The signal (μA) was calibrated in OD^{620} -units. The growth rate was determined from the growth curve in OD-units according to $\mu = \frac{d \log(\text{OD})}{dt} \cdot \frac{1}{\log e}$. Mostly logarithmic growth occurred just during the first half of the cultivation. It is this initial growth rate that is referred to in the further discussion.

Analyses. The amount of methane, nitrogen, and air were measured with precision flowmeters (Fisher & Porter) before mixing into the gas reservoir. The concentrations of methane, oxygen, and nitrogen in the mixture were measured before and after the cultivation with a gas chromatograph (thermistor detector, Molecular Sieve 5 A). pH of the medium was measured before and after each cultivation. Morphological studies were made with phase contrast microscope. Production of capsule was estimated by microscopic examination of cells suspended in nigrosin suspension. Amino acid analysis for lysin, methionine, cystin + cystein, threonine, tryptophan, and tyrosine were made according to Bolinder (186) on a medium from which cells had been separated by centrifugation and filtration. Absorption spectrum at 190 - 800 nm was made on the same cell free medium with a scanning spectrophotometer.

Isolation experiments. Isolation experiments were made on agar plates, agarose plates, and silica gel plates containing the same medium as described above. The silica gel plates were prepared from a colloidal dispersion of silica (Ludox). 240 ml of Ludox were mixed with 720 ml of water in which nutrients for 1 l medium were dissolved. The mixture was autoclaved during 20 minutes, cooled slowly to room temperature and pH adjusted to 6.0 with sterile 1 M HCl. The medium was then poured into petri dishes which were incubated in 60°C over night.

Inoculated plates were incubated at 30°C in a box containing 0.5 atm of methane and 0.5 atm of air. In the case of pressure incubation the plates were placed in a pressure cylinder (700 mm x Ø 70 mm) which was pressurized with 5.5 atm of methane and 0.5 atm of air. In the experiments, where liquid cultures were performed in this pressure cylinder, the culture was furnished with a magnetic stirrer (teflon coated rod).

Results

TM 10. Typical growth curves as recorded by the cultivation equipment and recalculated in OD-units are shown in fig. 3 on page 46. Two series of experiments were performed at 1 and 4 atm total pressure respectively. The effect of oxygen pressure on the specific growth rate shown in fig. 1. There was an evident maximum growth rate at about 0.2 atm of oxygen. Both lower as well as higher oxygen pressure reduced the growth rate. At about 2-3 atm of oxygen no growth occurred at all. When the oxygen pressure in the experiment with 2.8 atm of oxygen was released to 0.7 atm by reduction of the total pressure from 4 to 1 atm growth started very slowly (fig. 2) and did not reach the rate and cell density as did the control experiment.

The response to increased methane pressure shows that a slightly increased growth rate can be achieved by increasing the pressure from 0.5 to 1 atm (fig. 3). A decrease in methane pressure below 0.5 atm seems to drastically reduce the growth rate. In order to evaluate the kinetics of this reaction a Lineweaver - Burk plot was calculated on basis of the mean values of the experiments in fig. 3. This plot is shown in fig. 4. From this graph the maximum specific growth rate was estimated to be 0.153 h^{-1} and the saturation constant $K_s = 0.08 \text{ atm}$.

The final cell density was similar in most experiments and no relation was found between this parameter and the partial pressure of methane or oxygen. However, the final cell density was directly proportional to the pH decrease. In the cultures which had grown to the highest optical density (0.360) pH had dropped to 4.0. Certainly this explains the subsequently decreasing growth rate. The lag phase was mostly 1 - 1.5 hours and in a few experiments longer, but there was no evident relation between lag phase and oxygen or methane pressure.

Microscopic examination of the cultures before and after pressure cultivation did not reveal any changes in the relation between different morphological types during the cultivation. The amino acids analyses and the absorption spectra did not show any signs of leakage of metabolites from the cells.

Isolation experiments. Efforts were made to isolate pure cultures of methane oxidizing strains from TM10. Silica gel plates and agar plates were inoculated with serial dilutions of a grown out culture of TM10 and incubated in pure air and in 0.5 atm methane + 0.5 atm air. This comparison revealed the complex composition of the mixed culture. On agar plates there was a rich growth of colonies of several types grown out in air as well as in methane-air. On silica gel plates no or very sparse growth occurred on plates incubated in air and in methane-air a very few numbers and types of colonies appeared as compared to the agar plates. The yield of colonies on silica gel was about 1% of the yield on agar plates when incubated in methane-air. This led to the hypothesis that the agar used contained growth factors that admitted growth without methane. Agarose plates were used to test this. On agarose growth was generally more sparse than on agar but yet more rich than on silica gel (fig. 5).

Since elevated methane pressure had a stimulatory effect on the growth of TM10, experiments were made to check if it was possible to recognize the methane oxidizers from other strains by incubation under elevated methane pressure. Fig. 5 shows that the pressure incubated (5.5 atm methane + 0.5 atm air) silica gel plates yielded thicker colonies than did the silica gel plates in 0.5 atm methane + 0.5 atm air. On the incubated silica gel plates very small colonies appeared, but these were not able to reproduce when reinoculated under the same conditions. The agar plates did not show any remarkable differences between incubation in high pressure methane and 0.5 atm methane and when incubated in pure air growth was just somewhat poorer than in methane.

The strain isolated from TM10 that yielded the heaviest growth on silica gel was purified by repeated reinoculations on this medium. This strain, TM02, consisted of immotile rods ($0.8 \times 1.5 - 2 \mu$). The colonies on silica

gel appeared after 2 - 3 days and continued to enlarge till the branches covered almost all the plate. On agar plates the colonies did not spread so widely and they were much thinner and grey (fig. 6). This strain did not grow on methanol or nutrient broth, which was used as an indication that it was a pure strain of methane oxidizing bacteria.

A consecutive series of liquid cultures were made with TM02. This resulted in a gradually decreasing growth rate for each culture (fig. 7). Finally this strain died in the liquid line (after 7 reinoculations) as well as in the silica gel line (after six months). When the strain was kept on agar plates it gradually changed its characteristics, but it never regained the ability to grow in liquid culture on methane.

Studies were also made on the enrichment of methane oxidizing bacteria by incubation under 5.5 atm of methane. A 10 grams mud sample from a lake (Brunnsviken) was inoculated into 100 ml medium and incubated in 50% methane in air. This culture, which after 10 days showed dense turbidity, was used to inoculate the first pressure culture at 5.5 atm methane + 0.5 atm air in the stirred flask in the pressure cylinder. A series of consecutive reinoculations were made from these cultures. A similar control series was cultivated in 0.5 atm methane + 0.5 atm air. The growth in these experiments is shown in fig. 8. It is obvious that the pressurized cultures reached high cell density quicker than did the controls. When these cultures after 1 day, were inoculated on silica gel plates and incubated in 0.5 atm methane + 0.5 atm air no difference could be detected between the high pressure enriched cultures and the control with respect to total number of colonies per ml inoculum or the distribution of different colony types.

Discussion

The deleterious effect of high oxygen pressure on the growth was expected. The maximum partial pressure of oxygen that permitted initiation of growth of TM 10 seems to be in the order of 2 atm, a result which is consistent with many other reports concerning other bacteria (98). The long lag phase found after the release of the high oxygen pressure might be explained by the fact that high oxygen pressure is not only inhibitory to growth but may also be bactericidal (102). It is reasonable to suppose that many bacteria died during the 24 hours exposure to 2.8 atm of oxygen. The poor

growth that followed after the long lag phase may be explained by the complex composition of this culture. The different strains probably have different sensitivities to high oxygen pressure and a prolonged exposure to high oxygen pressure was likely to change the composition of the culture therefore, yielding a less adapted culture for growth under normal conditions.

The adverse effect of oxygen pressures below 0.2 atm seems a little more difficult to explain. According to literature (95) the K_s for aerobic bacterial growth is 0.001 - 0.01 atm O_2 and it is a common experience in many laboratories that oxygen does not limit the growth rate until its concentration is in the order of 0.02 atm or lower (corresponding to 10% air saturation). The closed system of cultivation that was used here did not permit replacement of the gas phase during cultivation so the oxygen and methane contents decreased and the carbon dioxide content increased during the cultivation. This was checked by gas chromatographic analyses prior to and after the cultivation. The mean decrease of oxygen partial pressure during the whole cultivation according to these analyses was 30%. The corresponding figure for methane was 37%. This means that the partial pressures during the last part of the growth curve were considerably lower than the figures used in fig. 1 and 3. On the other hand the growth was exponential only during the first part (40 to 70%) of the growth curve and this is the period from which the specific growth rates were calculated. Another possible reason for the oxygen limitation might be an insufficient gas transfer rate. However, the oxygen consumption rate must have been very low because of the low cell density. To investigate the effect of oxygen at these lower pressures one must use an equipment that offers the possibility for direct measurement of dissolved oxygen during growth.

The cultivations under increased methane pressure reveal that this culture responds according to the Michaelis - Menten model. The value of μ -max calculated from the Lineweaver-Burk plot is 0.153 h^{-1} and the methane pressure needed for this was at least 1 atm. This culture has earlier been grown in a turbidostat with μ -max 0.159 h^{-1} , but then the methane pressure was just 0.07 atm (173). The results from the turbidostat were achieved under quite different conditions e.g. higher cell density and higher mass transfer coefficients making it difficult to compare the values. Also in the investigation of the effect of methane one must keep in mind that the methane pressures used in fig. 3 and

4 are the initial pressures which means that the absolute values of the figures should not be emphasized but rather the tendency of the response to increased methane pressure.

There are few results in the literature about the effects of methane pressure on growth of methane oxidizing bacteria. In an US patent application (187) it is claimed that an increase in methane pressure to a few atm yields quicker growth. In a Japanese work (188) increased growth rate of a methane oxidizing bacteria was reported when the methane content in flask cultures were decreased from 60% to 10% in air. On the other hand, the same culture increased its growth rate both under the first logarithmic growth phase and under the remaining linear growth when the total gas pressure was increased from 1.2 to 3 atm total pressure. However, no figures on the methane content were given so the partial pressures were unknown. The author added the remark that increased fermenter pressure from 1 atm to 3 atm gauge pressure was not effective.

In this work with TM10 and in the two works discussed above one cannot exclude influences on the results by gas transfer effects on increased pressure because the dissolved methane concentration was not measured. There are two other investigations made with a completely different technique which measures the effect of methane concentration on the respiration rate (189, 190). In these experiments samples were taken from a methane limited chemostat and diluted with aerated methane free medium whereafter the dissolved oxygen uptake rate was measured at different methane concentrations. The K_s values for methane oxidation by two bacteria (Pseudomonas sp and Hyphomicrobium sp) were in the order of magnitude 20 μM . This corresponds to a methane pressure of approximately 0.01 atm in equilibrium with pure water or 1% methane in air at normal pressure and temperature. Besides by the differences in techniques used, the discrepancy between the reported K_s value for respiration (0.01 atm) and the value for growth (0.08 atm) that was found in this investigation might also be explained by the complex situation that is prevalent in a mixed culture. Methane is the only ultimate energy source for growth but it is partly converted into methanol and possibly other compounds which are excreted and reabsorbed by other strains. These reactions also take part in the total reaction, growth of the complete culture, that was measured in this investigation.

There is another factor that might be suspected to have influenced growth in these experiments and that is the carbon dioxide concentration. Carbon dioxide fixation has been shown to be involved in the metabolism of methane oxidizing bacteria (191, 192) and the total gas pressure will influence the dissolved carbon dioxide concentration. Very low (188) and very high (193) carbon dioxide concentrations may reduce growth and it would therefore be possible that the carbon dioxide pressure might have influenced growth in this investigation. The only effort to exclude this effect was that the initial carbon dioxide pressure in each culture was kept within $1 - 8 \cdot 10^{-4}$ atm. This concentration of dissolved carbon dioxide ought to have increased during the growth but the growth rate stabilized early on in each culture.

The results of the isolation experiments on TM 10 are in accordance with the experiences of many other authors. It is difficult to isolate pure strains and many strains cannot grow or grow very slowly in pure culture (176, 193, 194). One reason for lacking success in isolation of pure cultures is given by Harrison and Wilkinson (189, 190) who showed that the methane oxidizers in a mixed culture produced methanol that grew to inhibitory levels. The culture also contained methanol utilizing bacteria which grew on this methanol and therefore enabled further growth of the methane oxidizers.

The experience that many bacteria found in earth or mud are able to grow on components in agar is also in accordance with other reports (195). This constitutes a severe but perhaps not so well known problem in the isolation procedure.

The introduction of high pressure cultivation technique in the isolation of methane oxidizing bacteria did not prove to offer any immediate advantages even if the enrichment cultures grew faster under pressure and at least one methane oxidizing strain formed thicker colonies which developed faster than the controls in ordinary methane-air mixture. The fact that no change was found in the relation of the number of cells of different strains makes it plausible that the methane oxidizers as well as their commensals increased their growth rate when pressure was applied.

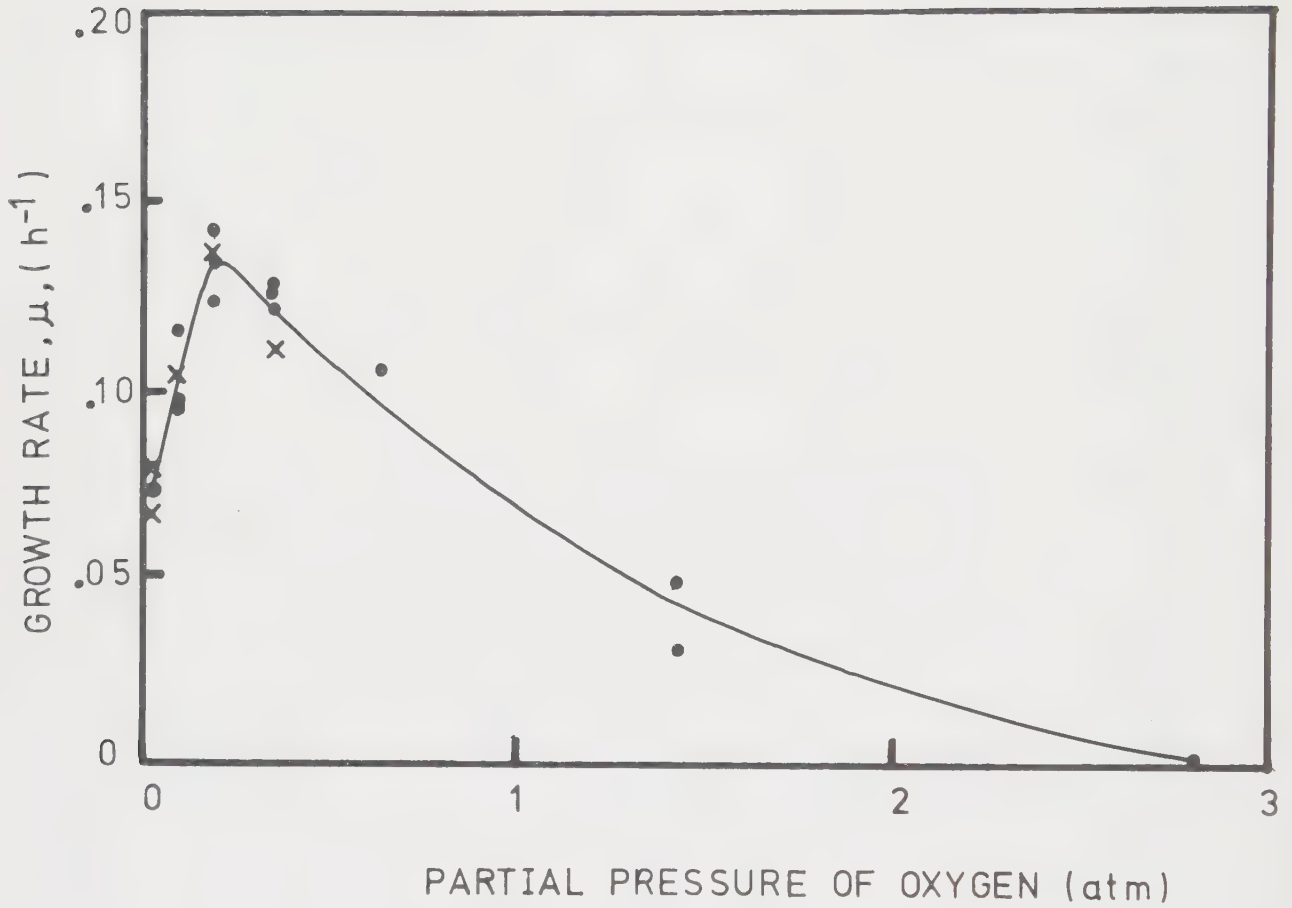


FIG 1. EFFECT OF PARTIAL PRESSURE OF OXYGEN ON THE SPECIFIC GROWTH RATE OF METHANE OXIDIZING BACTERIA (TM 10). THE PARTIAL PRESSURE OF METHANE WAS 0.6 ATM IN ALL EXPERIMENTS. THE TOTAL GAS PRESSURE WAS 1 ATM (x) OR 4 ATM (•).

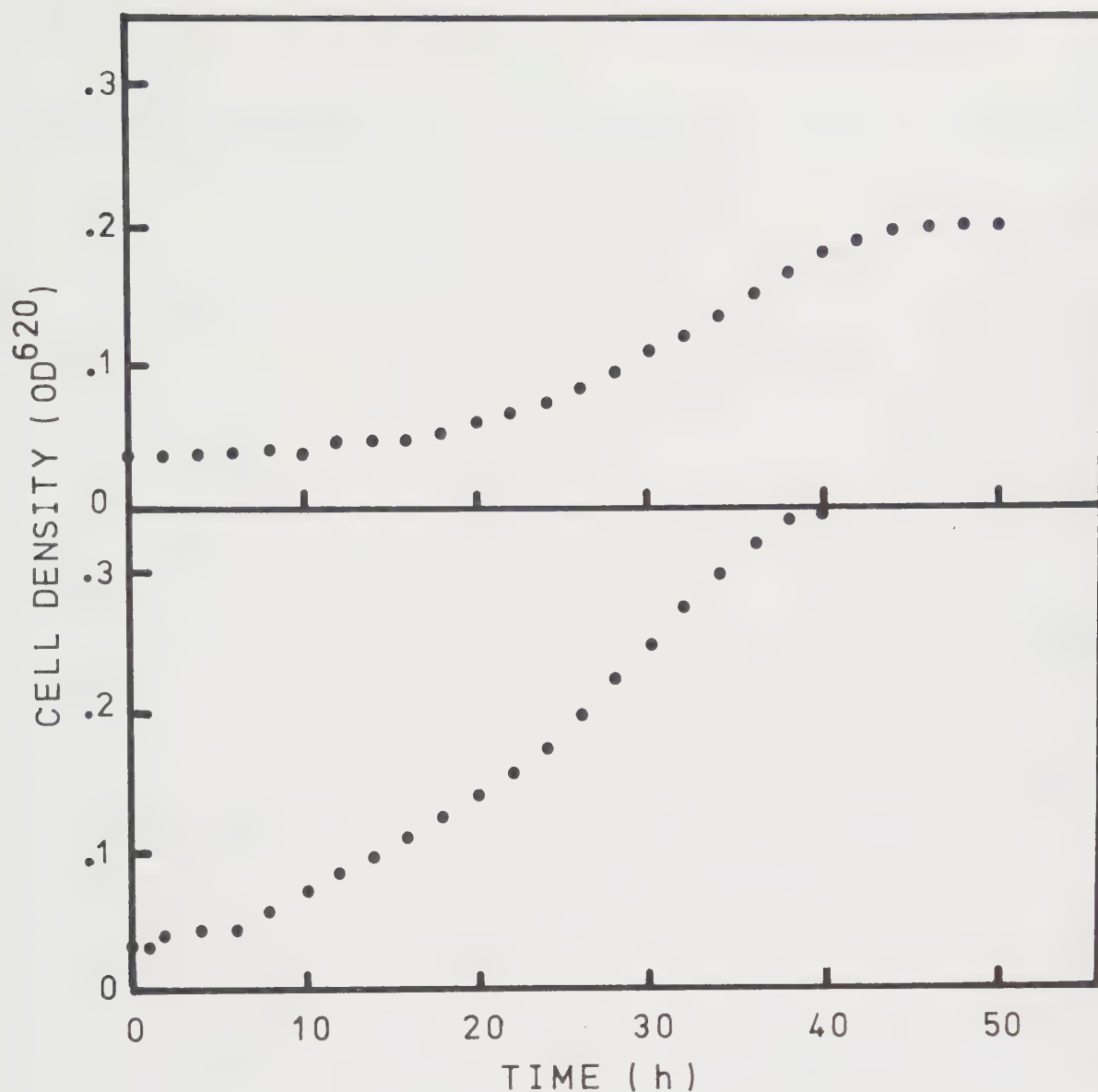


FIG 2. THE EFFECT OF EXPOSURE OF THE CULTURE TM 10 TO 2,8 ATM OF OXYGEN DURING 24 HOURS. THIS OXYGEN PRESSURE INHIBITED THE GROWTH COMPLETELY. WHEN THE PRESSURE WAS RELEASED TO 0,7 ATM OF OXYGEN GROWTH COMMENCED AND PROCEEDED AS SHOWN BY THE UPPER CURVE. THE LOWER CURVE SHOWS THE GROWTH OF A CONTROL AT 0,7 ATM OF OXYGEN. THE METHANE PRESSURE WAS 0,3 ATM IN BOTH EXPERIMENTS.

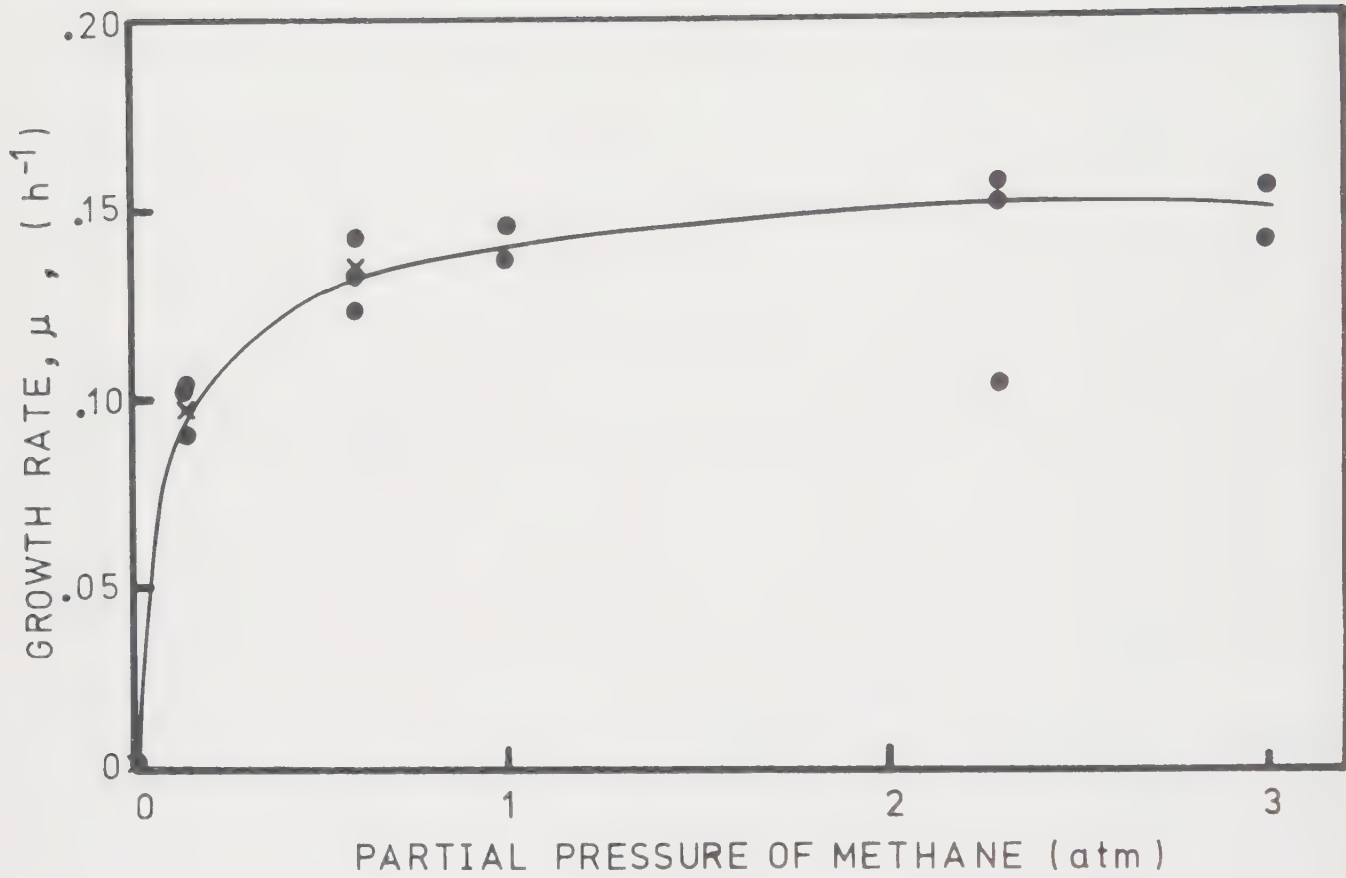


FIG 3. THE EFFECT OF THE PARTIAL PRESSURE OF METHANE ON THE SPECIFIC GROWTH RATE OF METHANE OXIDIZING BACTERIA (TM 10). THE OXYGEN PARTIAL PRESSURE WAS 0,2 ATM IN ALL EXPERIMENTS. THE TOTAL GAS PRESSURE WAS 1 ATM (x) OR 4 ATM (•).

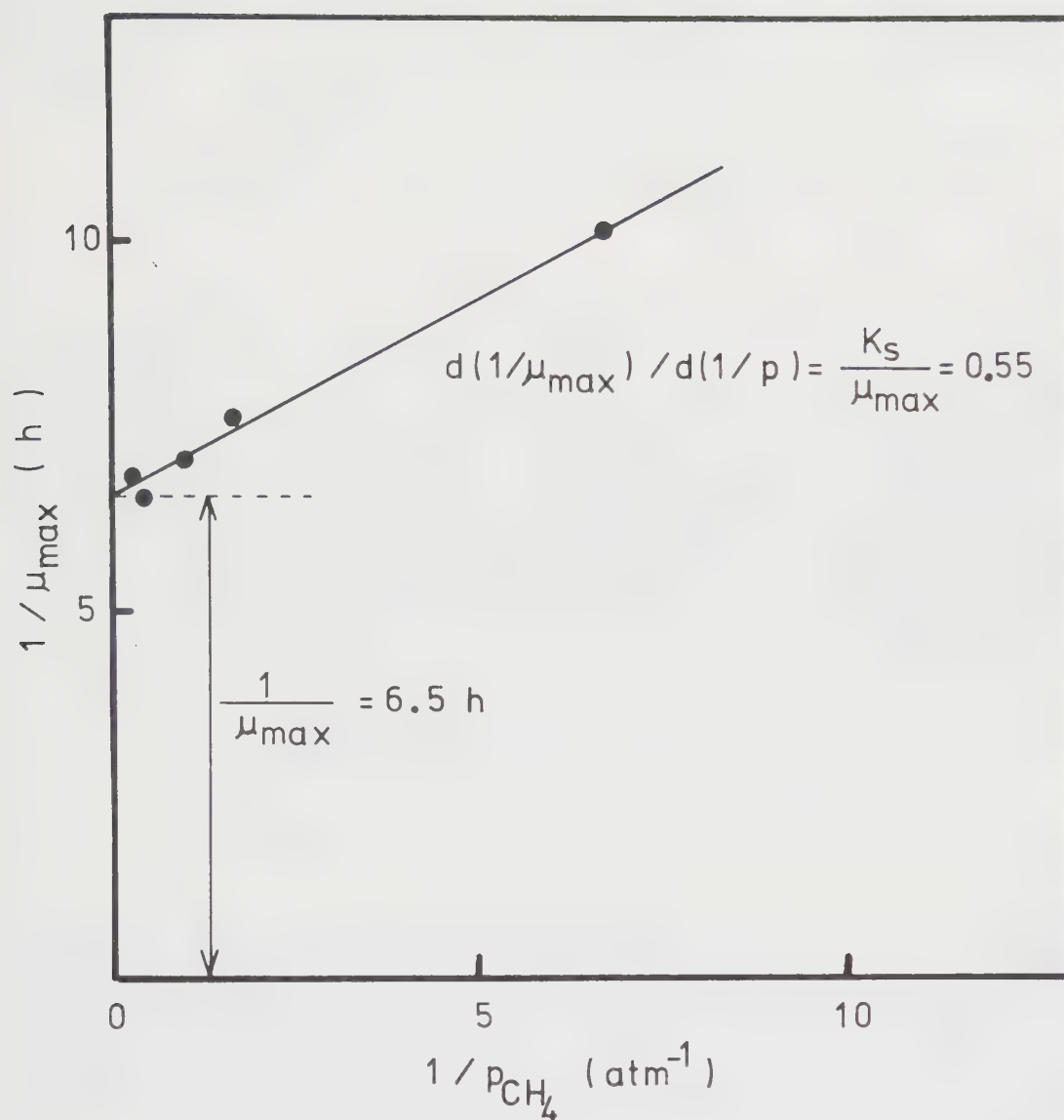


FIG 4. A LINEWEAVER-BURK PLOT OF THE DATA IN FIG 3. $\mu_{\max}$ WAS CALCULATED TO 0.153 h^{-1} AND K_s TO 0.08 ATM .

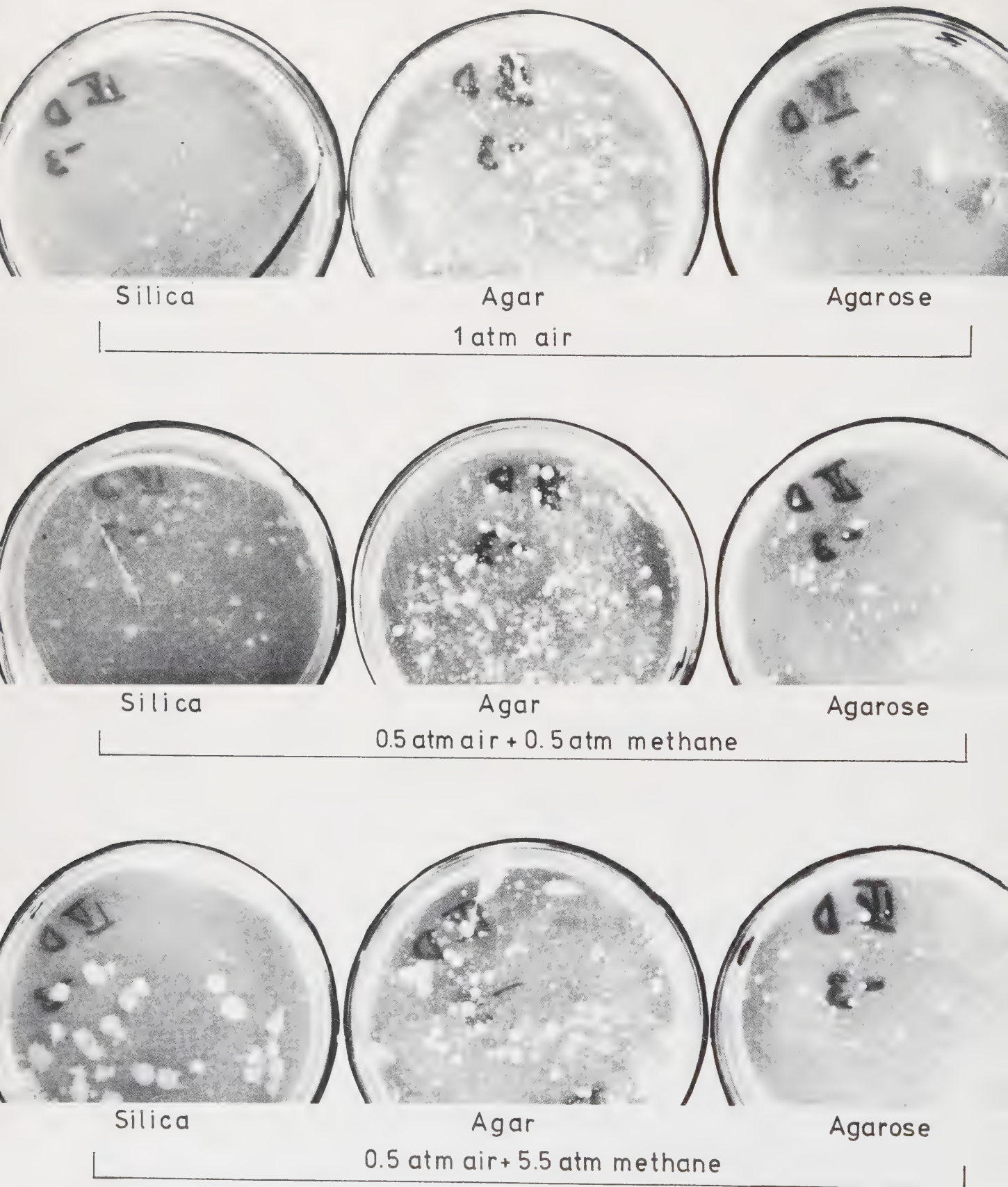


FIG 5. A COMPARISON BETWEEN DIFFERENT MATERIAL FOR SOLID MEDIA FOR GROWTH OF METHANE OXIDIZING BACTERIA (TM 10). THE PLATES WERE INCUBATED IN PURE AIR OR IN A 50 - 50 MIXTURE OF METHANE-AIR AT 1 ATM OR IN A PRESSURE VESSEL WITH 0,5 ATM OF AIR AND 5,5 ATM OF METHANE. INCUBATION TIME 0 DAYS. SCALE 1:1

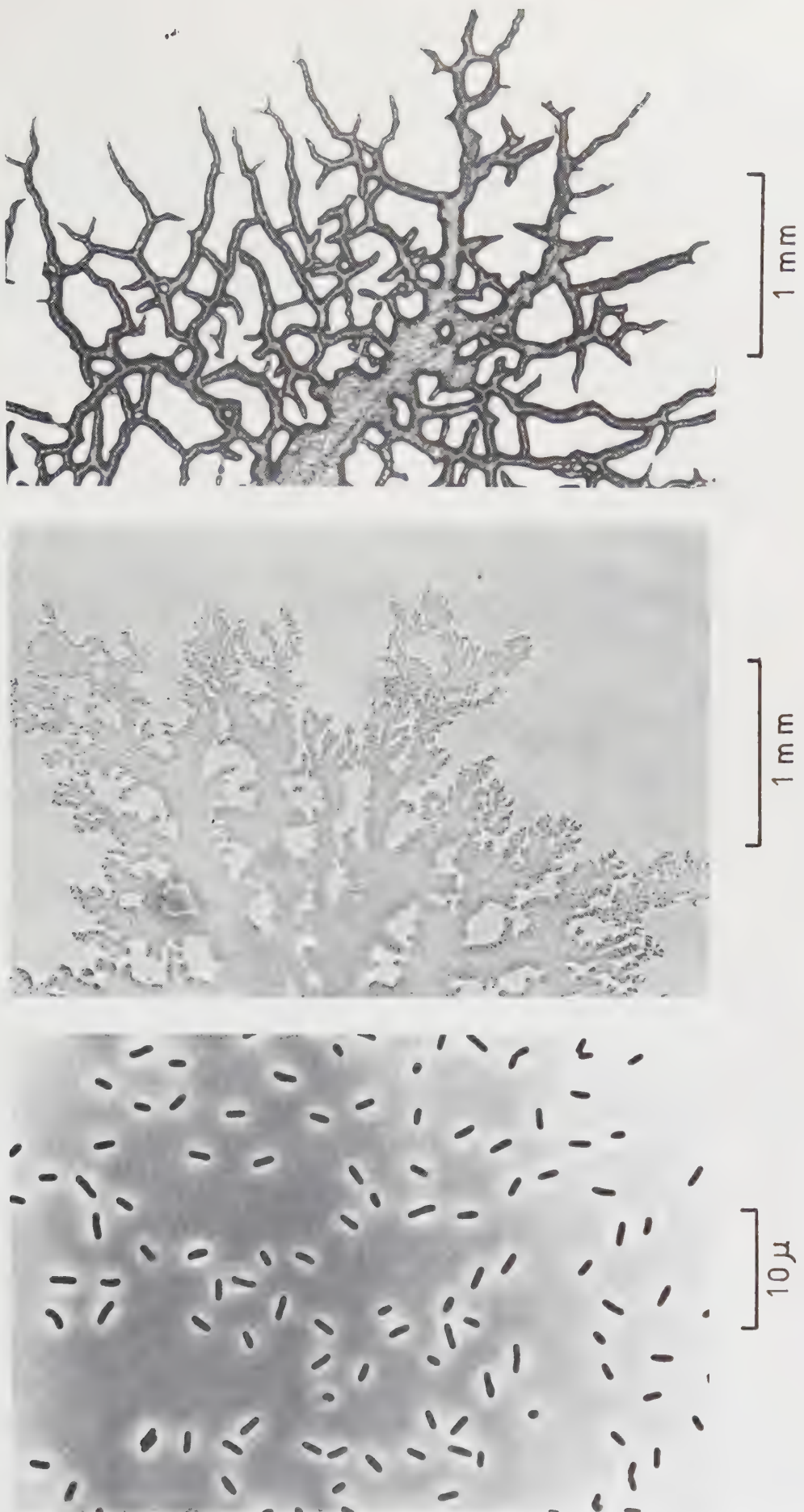


FIG 6. THE METHANE OXIDIZING BACTERIA TM 02 INCUBATED DURING 16 DAYS ON A SILICA GEL (UPPER PICTURE) AND ON AGAR (MIDDLE PICTURE). THE LOWER PICTURE SHOWS A MICROPHOTO OF A LIQUID CULTURE AFTER 5 DAYS.

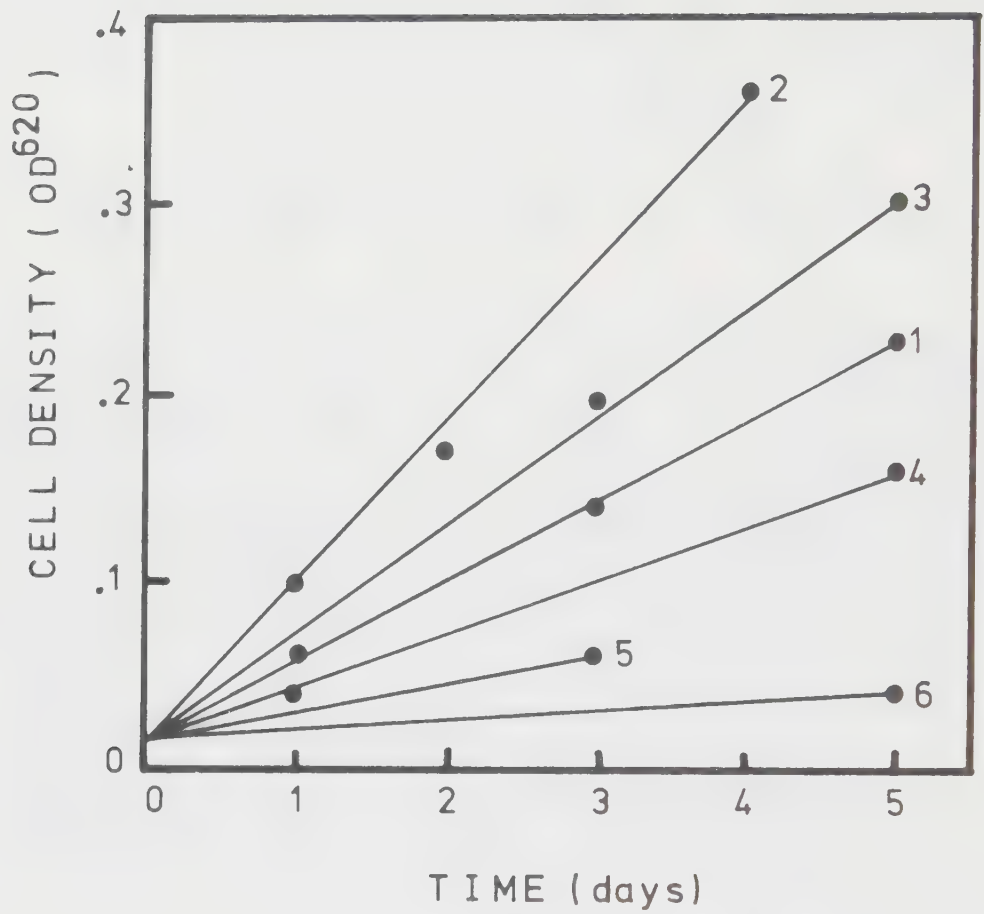


FIG 7. GROWTH OF THE METHANE OXIDIZING STRAIN TM 02 IN LIQUID MEDIUM. A CONSEQUITIVE SERIES OF CULTIVATIONS WAS MADE. THE NUMBER SHOWS THE ORDER OF THE CULTIVATION. AFTER 6 CULTIVATIONS THE ORGANISM HAD LOST ITS CAPABILITY TO GROW IN THIS MEDIUM.

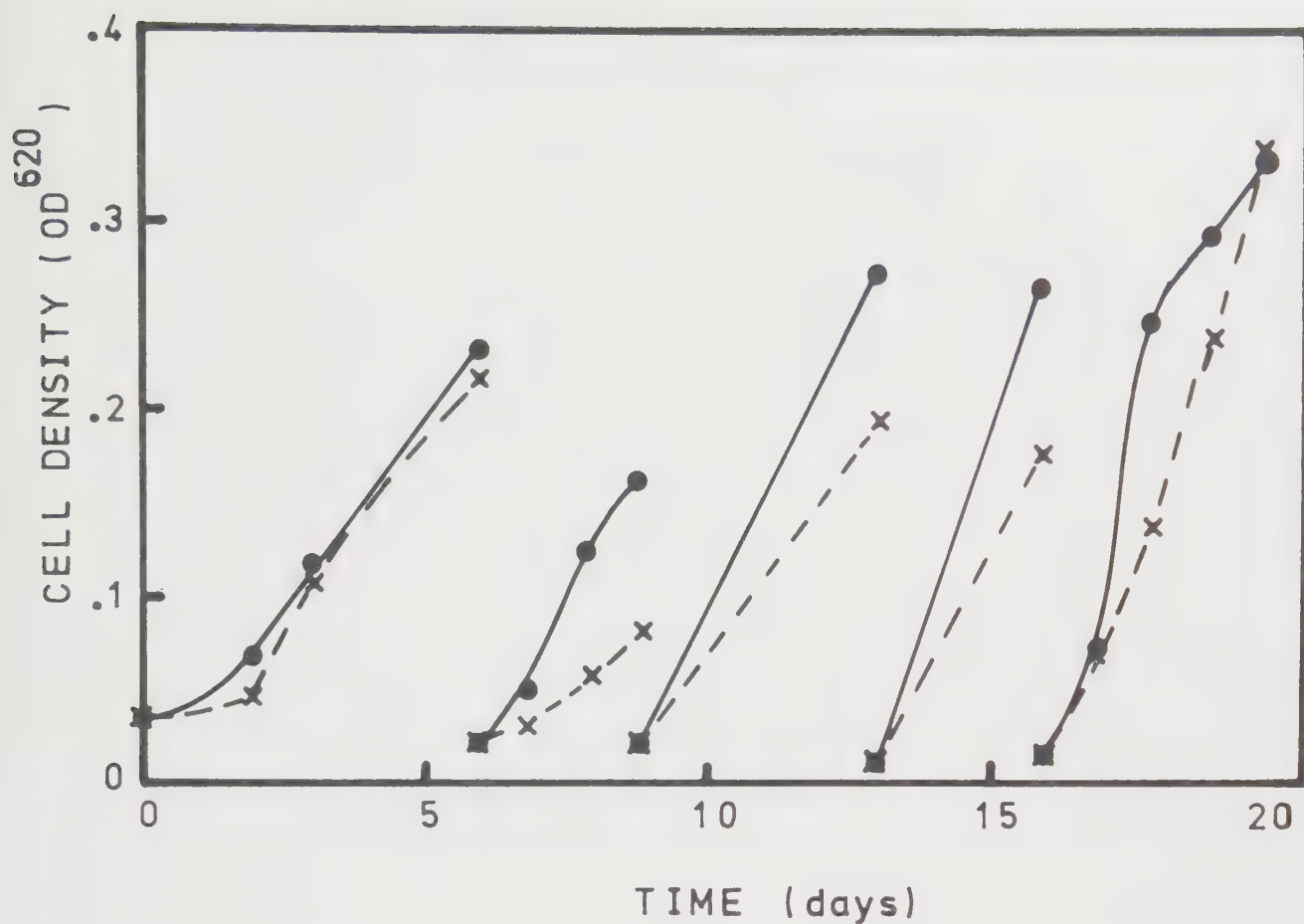


FIG 8. ENRICHMENT CULTURES OF METHANE OXIDIZING BACTERIA CULTIVATED IN 0,5 ATM OF AIR + 5,5 ATM OF METHANE (•) AND 0,5 ATM OF AIR + 0,5 ATM OF METHANE (x). AT THE END OF ONE CULTIVATION THE CULTURE WAS USED TO INOCULATE THE NEXT CULTURE FOR INCUBATION IN SIMILAR ATMOSPHERE.

EFFECTS OF HIGH AIR PRESSURE ON BACTERIAL GROWTH, SPORULATION AND SPORE GERMINATION

Summary

The effects of elevated air pressure on some basic microbial reactions were investigated. The growth rate of Escherichia coli was not affected by 4 or 6 atm of air but an initial death of some of the cells was found immediately after incubation in air pressure. The lag phase and growth rate of Bacillus stearothermophilus were not influenced by air pressure up to 3 atm. At 5 atm of air the lag phase became longer and the growth rate became slower. At 7 atm of air no growth occurred at all. No adaptation to high air pressure was found. The sporulation of this organism was a little more susceptible than was growth. In Bacillus cereus growth and sporulation were equally sensitive to high air pressure. Inhibition was evident at 3 atm of air and it was reversible. The germination of B. cereus spores was not influenced by 10 atm of air but 50 atm of air caused some inhibition.

Introduction

The inhibitory and toxic effects of high oxygen pressure (HPO) on almost any organism are well documented though no theory on the mechanism of this phenomenon is generally accepted. Experiments with microorganisms support the view that the high oxygen tension generates too high a concentration of peroxides, which cannot be eliminated quickly enough by the cell. The catalase content of Bacillus subtilis, Saccharomyces cerevisiae and Candida utilis were shown to covariate with the resistance to high pressure of oxygen (121, 122, 197). However, more recent investigations focus the interest on the production of superoxide radicals which constitutes substrates of superoxide dismutases which catalyse the reaction $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$. Superoxide dismutases are ubiquitously prevalent in microorganisms with aerobic metabolism (105, 106, 107). In these works it was shown that oxygen may induce superoxide dismutase in bacteria and yeast and that the activity of these enzymes covariates with the resistance to oxygen. It was also shown that Bacillus subtilis gained catalase activity during exposure to high pressure oxygen but this did not result in increased resistance to oxygen toxicity. Nor did the superoxide dismutase activity increase.

Experiments with animals and many in vitro experiments maintain the opinion that the oxygen toxicity acts on the energy metabolism by oxidizing the sulfhydryl groups in some enzymes. Especially FAD-depending enzymes have proved to be sensitive (113, 117).

There is a large diversity of results on cultivation of microorganisms under elevated oxygen pressure (92, 93, 96, 98 rev., 117). Some organisms have been found to grow best in oxygen enriched atmospheres (92, 93). It is remarkable if some organisms should have an optimum at oxygen concentrations higher than corresponding 0.2 atm, since a lot of experience from dissolved oxygen controlled experiments shows that the saturation constant for growth on, as well as for respiration of, oxygen is very low, in the order of $10^{-2} - 10^{-6}$ atm and the relation between reaction rate and substrate concentration (P_{O_2}) shows the same pattern for oxygen as for other macroelement substrates as carbon, nitrogen or phosphorus (95, 198, 199, 200, 201).

There is a reasonable explanation to the reported stimulating effect of high oxygen tension. This deals with how one defines the sentence better growth. If better growth means growth to a higher cell density within a given time then it is possible that increased air pressure will result in better growth since many cultures are so dense and grow so fast that they consume the available oxygen in the medium. If the growth then continues under oxygen limitation this will result in a growth rate which depends on the oxygen transfer rate which under these circumstances is a direct function of the oxygen pressure. The conclusion of this is that the dissolved oxygen concentration ought to be measured when one wants to investigate the growth response to increased oxygen pressure. Otherwise one has to be careful and keep in mind that oxygen pressure is not synonymous to dissolved oxygen concentration in a dense culture that grows at a high rate. In analogy to this it is plausible to assume that there is an oxygen concentration gradient in a growing colony or an unstirred culture.

The aim of this work was to study the effect of increased air pressure on some basic microbial reactions viz. growth, sporulation, and spore germination. The growth was studied with respect to growth rate as well as to total amount of generated cells. Three types of organisms were chosen:

Escherichia coli B, Bacillus stearothermophilus, and Bacillus cereus.

The sporulation of the two Bacillus species was also studied at increased air pressure. Special attention was made to the possibility to synchronize this reaction by inhibiting the process at a hypothetical extra oxygen sensitive step followed by a transfer of the culture into ordinary conditions. It has been shown that cell division can be synchronized in that way if the culture is exposed to high oxygen pressure and then transferred to 1 atm of air (121).

It was also of interest to see if germination of spores, which is considered to be independant on energy metabolism, is sensitive to high air pressure since one of the theories on oxygen toxicity considers an interaction with some enzymes in the energy metabolism leading to ATP-deficiency to be the reason for the universal inhibitory effect of high oxygen tension on living organisms (202).

Materials and methods

Strains: Escherichia coli B was obtained from Department of Bacteriology, Karolinska Institutet, Stockholm and Bacillus cereus (SIK No. 216) and Bacillus stearothermophilus were obtained from SIK, Göteborg.

Media: E. coli B was cultivated at 37°C in FSA medium:

Na lactate 5 g/l, NH_4Cl , 1.0 g/l, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ 0.9 g/l, KH_2PO_4 0.3 g/l, Na_2SO_4 0.4 g/l, and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02 g/l.

Viable counts were made in the same medium with 1.5% agar. B. stearothermophilus was kept on TYG-medium: trypton 10 g/l, yeast extract 5 g/l,

K_2HPO_4 , 2 g/l, and glucose 5 g/l. The growth and sporulation studies under elevated air pressure were done in TRY-medium: trypton 20 g/l, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ 0.47 g/l, KH_2PO_4 0.18 g/l, and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.08 g/l. All cultiva-

tions were performed at 55°C. B. cereus was cultivated at 30°C in G-medium:

MgSO_4 8 g/l, $(\text{NH}_4)_2\text{SO}_4$ 4 g/l, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.1 g/l, K_2HPO_4 1 g/l, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g/l, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.1 g/l, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.1 g/l, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.01 g/l, yeast extract 2 g/l, glucose 4 g/l.

Cultivations: Inoculum of E. coli B was taken from a logarithmically growing shaking flask culture. $2.5 \cdot 10^9$ cells from this culture were inoculated into 25 ml medium in the high pressure cultivation equipment described on page 40. However, no gas reservoir was used but the top of the cultivation vessel was plugged with cotton wool. The pressure vessel was filled with compressed air at a rate of 3 atm/min. Each second hour the pressure was released and the pressure vessel was opened for sampling (0.2 ml). This sample was used for viable count by serial dilution and inoculation on agar plates and for microscopic counting in a Bürker-chamber. In each case the compression/decompression rate was kept at 3 atm/min. The sampling required another 2 minutes.

A 100 ml culture of B. stearothermophilus cultivated in a bottle with magnetic stirrer was used for inoculation into the pressure vessel. The inoculum was harvested in late growth phase and washed once in 0.1 M phosphate buffer pH 7.0. The pressure vessel used for these experiments was a 500 ml high pressure autoclave (RK105, Dr. Thiedig & Co KG) shown in fig. 1. The complete pressure vessel with 300 ml of fresh medium was sterilized in an autoclave. The inoculum was pumped into the pressure vessel with a peristaltic pump. The pressure vessel was furnished with a mixer consisting of two perforated discs which were run up and down with an amplitude of 50 mm and a speed of 60 RPM. A constant airflow (0.1 l/min.) was filtered into the culture. The outlet air valve was throttled to increase the pressure in the vessel. The complete pressure vessel was placed in a thermostated oven which kept the temperature at $55 \pm 1^\circ\text{C}$. Samples were taken out intermittently through one of the valves. 10 ml of the sample was centrifuged and the cells were resuspended to the original volume in 0.1 M phosphate buffer at pH 7.0. This sample was used for cell density measurements (OD^{620}). An untreated sample was used for direct counting of the frequency of spores in a phase contrast microscope.

B. cereus was cultivated in the same vessel as was used for B. stearothermophilus. 50 ml of inoculum, prepared in a shaking bottle, was used. Harvesting was made in late growth phase, and the inoculum was washed once in 0.1 M phosphate buffer at pH 7.0. The medium for B. cereus was almost colorless and growth could therefore be followed by direct measurement of OD^{620} . Sporulation frequency was calculated as was mentioned for B. stearothermophilus.

Germination studies: Spores of B. cereus were prepared by inoculation of 500 ml G-medium with a few colonies from an agar slant. This culture was incubated on a shaker and it sporulated to 95% within a few days. The spores which finally became free from sporeangium were washed 3 times in 0.067 M phosphate buffer at pH 7.0. The spores were finally suspended in the same buffer to $OD^{620} \sim 0.6$. 300 ml of this solution was heat activated at $80^{\circ}C$ for 15 minutes, then cooled under tap water and pressurized in the high pressure autoclave for one hour. During this time mixing was achieved by a slow airflow through the spore suspension. A control sample was then taken out and a germination medium consisting of a solution of L-alanine and adenosine was added to the control and the pressurized sample. The final concentrations were 10 mM L-ala and 2mM adenosine. The addition of germination medium to the pressurized sample was accomplished by starting the stirrer to which a fragile glass ampoule with the germination medium was attached. The germination was followed photometrically by absorbance measurement at 620 nm. The control was measured continuously and the data were recorded. The germination under pressure was followed by intermittent sampling. The germination in these samples was also verified by microscopic observation in a phase contrast microscope.

Results and discussion

Growth. The growth curves constructed from microscopic counts of E. coli B cultivated in 1, 4, and 6 atm of air do not show any evident differences (fig. 2). This technique for measurement of cell density has a limited accuracy, which makes it unwise to draw conclusions from small deviations. The growth curves from the built in photometer are shown in fig. 3. They show the same pattern as the growth curves from microscopic counts. However, in the case of growth curves from viable counts, (fig. 4) there is a striking effect of high air pressure. It is visible in the 4 atm diagram and still more evident in the 6 atm diagram that the viability of the culture decreased immediately after it had been subjected to high air pressure. It can also be seen that the viability of the culture exposed to high air pressure decreased as soon as it had reached its stationary state. The growth rates and the total number of produced cells calculated from the three methods to measure cell density are compared in Table I.

These results can be elucidated by two theories on oxygen toxicity. According to Gifford and Pritchard (121, 122) the resistance to H₂O₂ is related to the catalase activity. They found that the catalase activity in Escherichia coli, Bacillus subtilis, Saccharomyces cerevisiae and Candida utilis was highest immediately after the cell division and that this period coincided with the time of highest resistance to H₂O₂. If this is valid also for the culture used in this investigation, then it seems reasonable that one part of the population, the one which was close to the next cell division, had too small a catalase activity to withstand the increased oxygen pressure. The catalase activity of a culture has also been shown to increase when it is cultivated under H₂O₂ which makes it probable that these cells to some degree were able to adapt to H₂O₂-conditions (121, 197).

According to an other theory (105, 106, 107) it is the superoxide dismutase activity rather than the catalase activity that determines the resistance of the cell to high oxygen concentration. It has been shown that oxygen can induce increased activity of superoxide dismutase in a very short time (half response within 90 min) in Streptococcus faecalis and that the same induction occurs in E. coli B (105). Such a response might also be the explanation to the initial decrease in viable cells found in this investigation.

The sensitivity of the growth of B. stearothermophilus to high air pressure is shown in fig. 5 and table II. The growth rate was approximately constant at 1 - 3 atm of air but at 5 atm of air there was a marked reduction in growth rate and at 7 atm of air there was no growth at all.

Experiments were also performed to check if any adaptation occurred in the 5 atm culture when growth was so evidently inhibited. Cells of B. stearothermophilus were harvested in the end of the 5 atm growth curve and reinoculated in a new 5 atm-culture. This culture did not differ from the mother culture which shows that no adaptation to the high air pressure environment had occurred. (fig. 5). According to the investigations on the superoxide dismutase (105, 106, 107) many organisms were able to adapt to oxygen enriched environments by increasing their activity of superoxide dismutase. In those experiments the only Bacillus species tested, Bacillus subtilis, did not gain any increased resistance to oxygen nor activity of superoxide dismutase (106).

The cultures of B. cereus showed a similar response to HPO (fig. 6,7,8). In this case the decrease of the growth rate at pressures above 3 atm was evident. In the B. stearothermophilus experiments, however, the growth phase became longer when the air pressure was increased from 1 to 3 atm. This effect was not shown by the B. cereus cultures. The growth rate of B. stearothermophilus and the cell density were so low that the oxygen consumption can not be expected to reduce the DO-concentration considerably.

It is hard to compare these results with the results on E. coli B since the cultivation equipments were not identical. This means that for instance the oxygen transfer rate is an uncertain factor. The apparent higher resistance to high air pressure by E. coli B might be explained by a higher oxygen consumption rate resulting in a lower dissolved oxygen concentration. An induction of superoxide dismutase might also be the reason for the higher resistance.

Sporulation. The sporulation of B. stearothermophilus was more sensitive to high air pressure than was growth (fig. 9). At 3 atm there was an evident delay in sporulation and at 5 atm there was no sporulation at all though some growth could be stated. These result led to the following hypothesis. It is reasonable to believe that among a series of reactions leading to the spore, one of the reactions might be more oxygen sensitive than the others. Thus, if the whole population was stopped by high air pressure in this step and then the air pressure was released, there would be a possibility that the complete population simultaneously continued to mature into spores.

In order to test this hypothesis the more abundantly sporulating B. cereus strain was used. The hypothesis could however not be verified. When the pressure was released from 5 and 5.5 atm the sporulation did indeed increase, but at a slow rate and to an extent that was far from the sporulation in the control that had been cultivated at 1 atm (fig. 8). It was also evident from these experiments that the growth that had been stopped at a relatively low cell density by 5 or 5.5 atm recovered at pressure release. Similar results were obtained when the culture was allowed to grow out and the pressure was applied just before the sporulation.

Germination. The germination of B. cereus spores in high air pressure is shown in fig. 10. It can be concluded that germination is much more resistant to high air pressure than are growth and sporulation. 10 atm of air do not seem to influence the germination at all. At 50 atm of air, however, the inhibition seems to be evident. 50 atm of air exerts an oxygen pressure of 10 atm which is, according to available literature, highly toxic for living organisms (98). This treatment also causes a hydrostatic pressure of 50 atm which is of an order that could be suspected to influence life reactions. Reports on the effects of hydrostatic pressure on spore germination reveal however that it has a stimulating rather than inhibitory effect in the range of 200 - 1000 atm (64, 65, 66, 67). This phenomenon will be further investigated.

TABLE I.

METHOD	PARAMETER	AIR PRESSURE		
		1 ATM	4 ATM	6 ATM
MICROSCOPIC COUNTING	GROWTH RATE (μ) h^{-1}	1.65	1.69	1.73
	NUMBER OF PRODUCED CELLS/ML	$10^{10.0}$	$10^{10.0}$	$10^{9.8}$
VIABLE COUNT	GROWTH RATE (μ) h^{-1}	1.61	1.84	1.68
	NUMBER OF PRODUCED CELLS/ML	$10^{10.1}$	$10^{10.1}$	$10^{10.2}$
PHOTO- METER	GROWTH RATE ($\frac{d\mu A}{dt}$)	53	41	49
	NUMBER OF PRODUCED CELLS (FINAL μA)	230	220	210

CULTIVATION OF E. COLI B IN 1, 4 AND 6 ATM OF AIR. THE GROWTH RATE AND THE FINAL AMOUNT OF CELLS ACCORDING TO THREE METHODS OF ANALYSIS ARE COMPARED. ONE CULTIVATION WAS PERFORMED AT EACH PRESSURE.

TABLE II.

AIR PRESSURE (ATM)	GROWTH RATE $\frac{d OD}{dt}$ (DAY ⁻¹)	CELL YIELD OD _F - OD _I
0	0	0
1	0,5	0,20
2	0,52	0,21
3	0,40	0,25
5	0,06	0,06
5 ^x)	0,08	0,07
7	0	0

CULTIVATION OF B. STEAROTHERMOPHILUS IN 0 - 7 ATM OF AIR.
 IN THE 0 ATM EXPERIMENT THE CULTURE WAS FLUSHED WITH NITROGEN.
 THE LINEAR GROWTH RATE IS EXPRESSED AS THE SLOPE OF THE CURVE
 AND THE CELL YIELD IS EXPRESSED AS THE DIFFERENCE BETWEEN THE
 FINAL AND THE INITIAL CELL DENSITY VALUES. EACH FIGURE, EXCEPT
 THOSE OF THE ^x)-MARKED EXPERIMENT, IS A MEAN VALUE OF TWO
 EXPERIMENTS. THE ^x)-MARKED EXPERIMENT WAS PERFORMED WITH
 INOCULUM FROM A 5-ATM CULTURE.

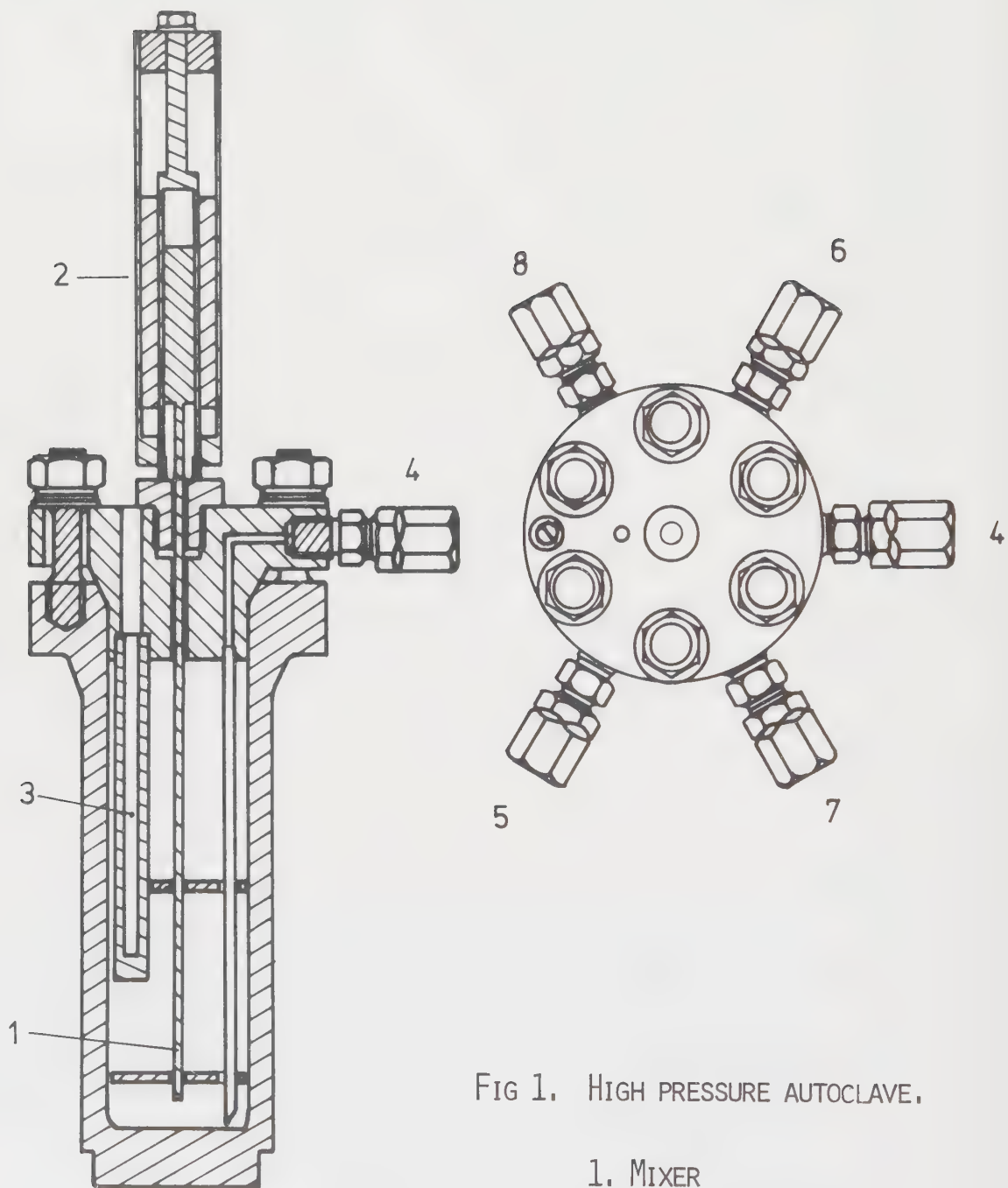


FIG 1. HIGH PRESSURE AUTOCLAVE.

- 1. MIXER
- 2. MAGNETIC COUPLING
- 3. THERMISTOR POCKET
- 4. GAS INLET
- 5. GAS OUTLET
- 6. SAMPLING
- 7. MANOMETER
- 8. PRESSURE RELEASE VALVE

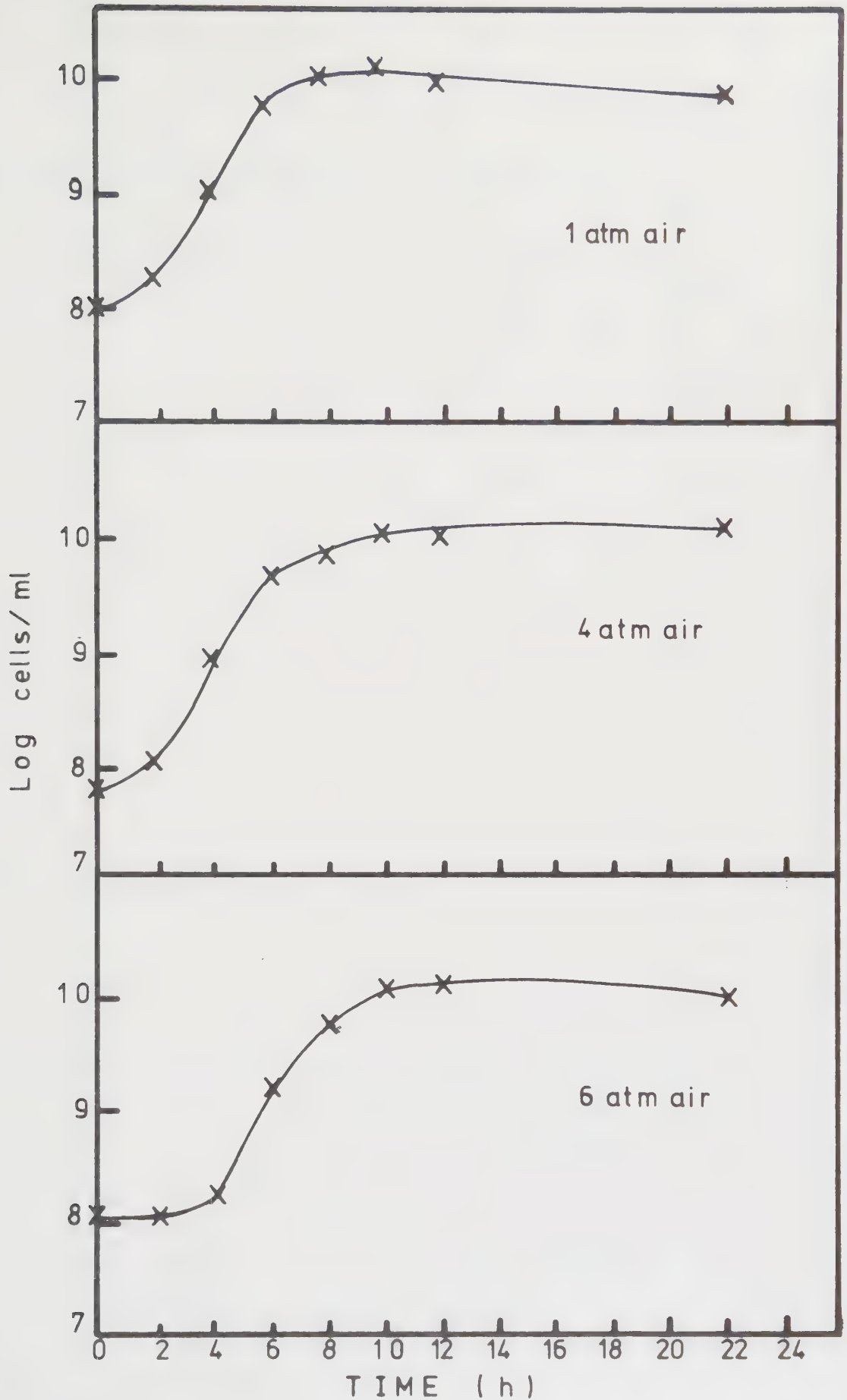


FIG 2. GROWTH OF *E. COLI* B IN 1, 4 AND 6 ATM OF AIR. DATA FROM MICROSCOPIC COUNTING IN BÜRKER-CHAMBER.

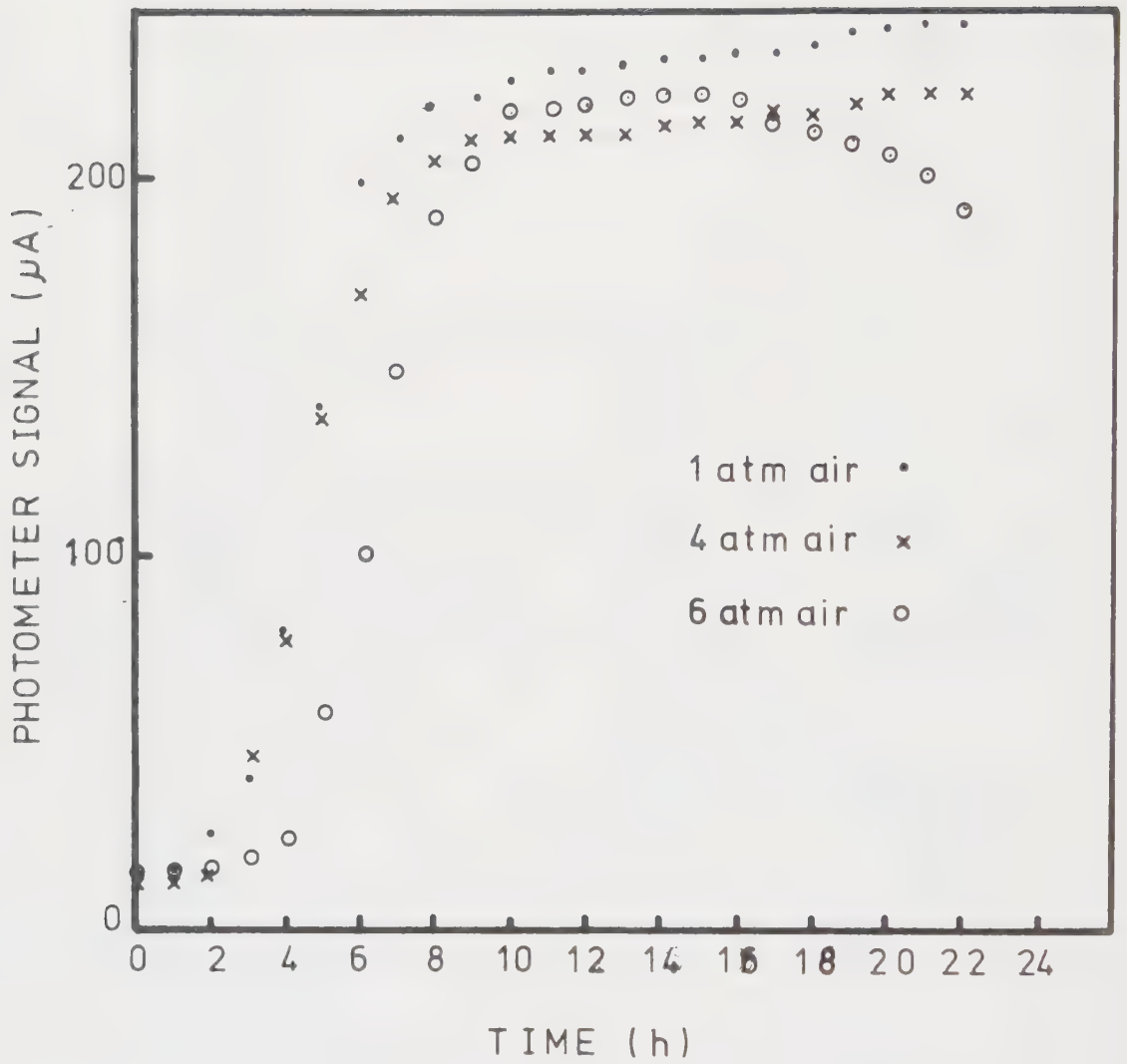


FIG 3. GROWTH OF *E. COLI* B IN 1, 4 AND 6 ATM OF AIR. DATA FROM THE BUILT IN PHOTOMETER.

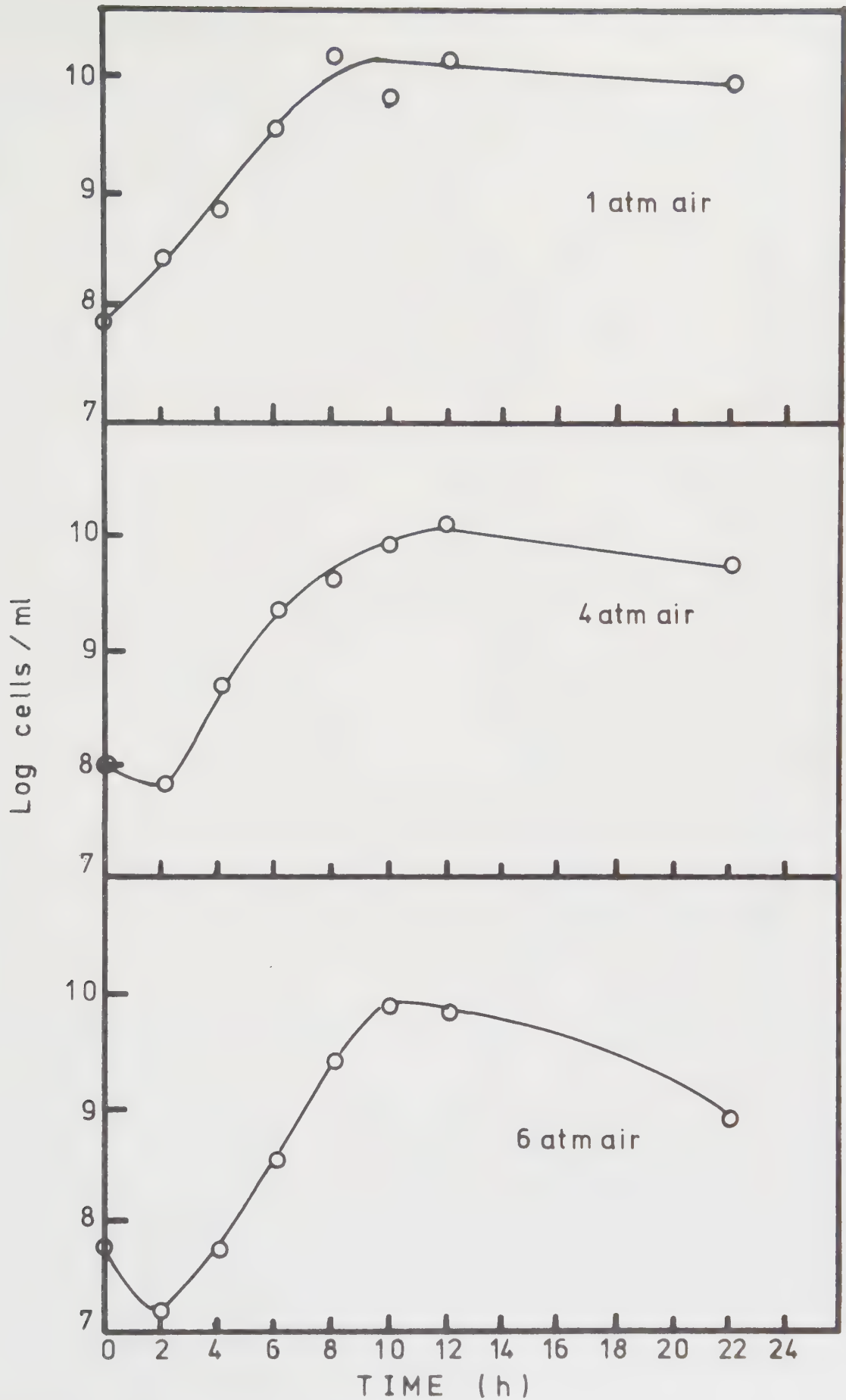


FIG 4. GROWTH OF *E. COLI* B IN 1, 4 AND 6 ATM OF AIR, DATA FROM VIABLE COUNTS.

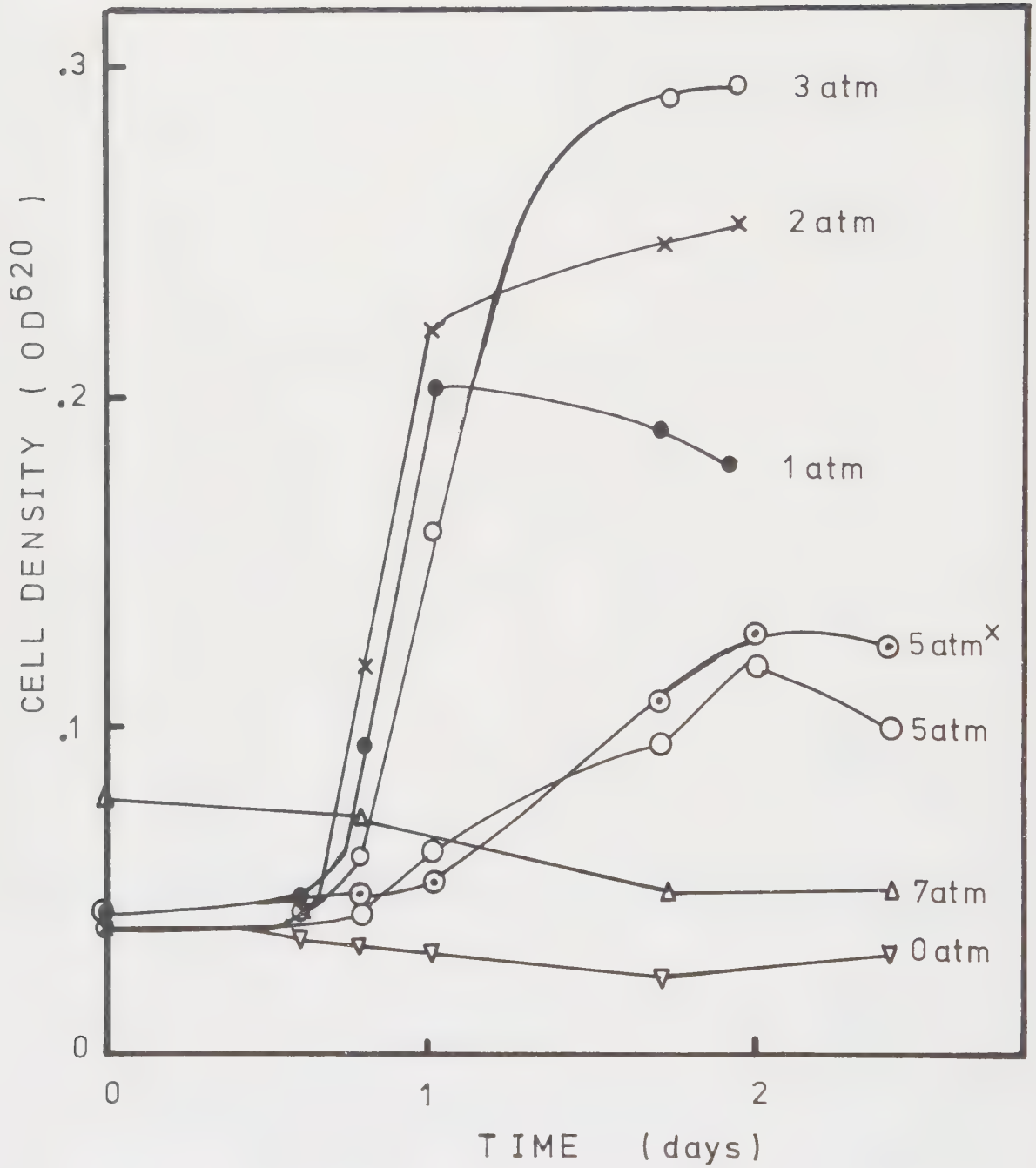


FIG 5. GROWTH OF *B. STEAROTHERMOPHILUS* IN 0 - 7 ATM OF AIR. THE CULTURE MARKED WITH X) WAS INOCULATED WITH CELLS FROM THE END OF THE OTHER 5 ATM-CULTURE.

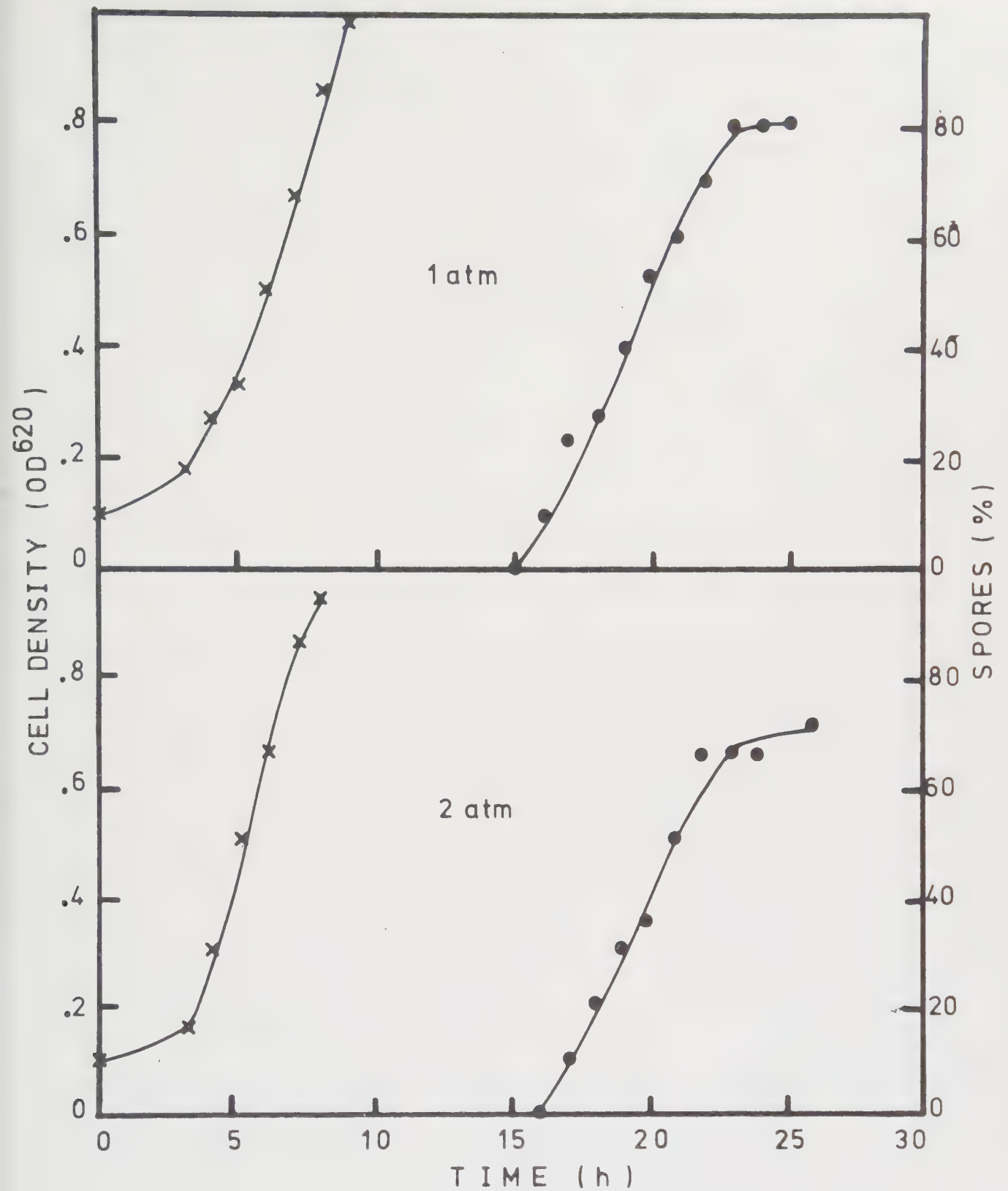


FIG 6. GROWTH (x) AND SPORULATION (•) OF *B. CEREUS* IN 1 AND 2 ATM OF AIR.

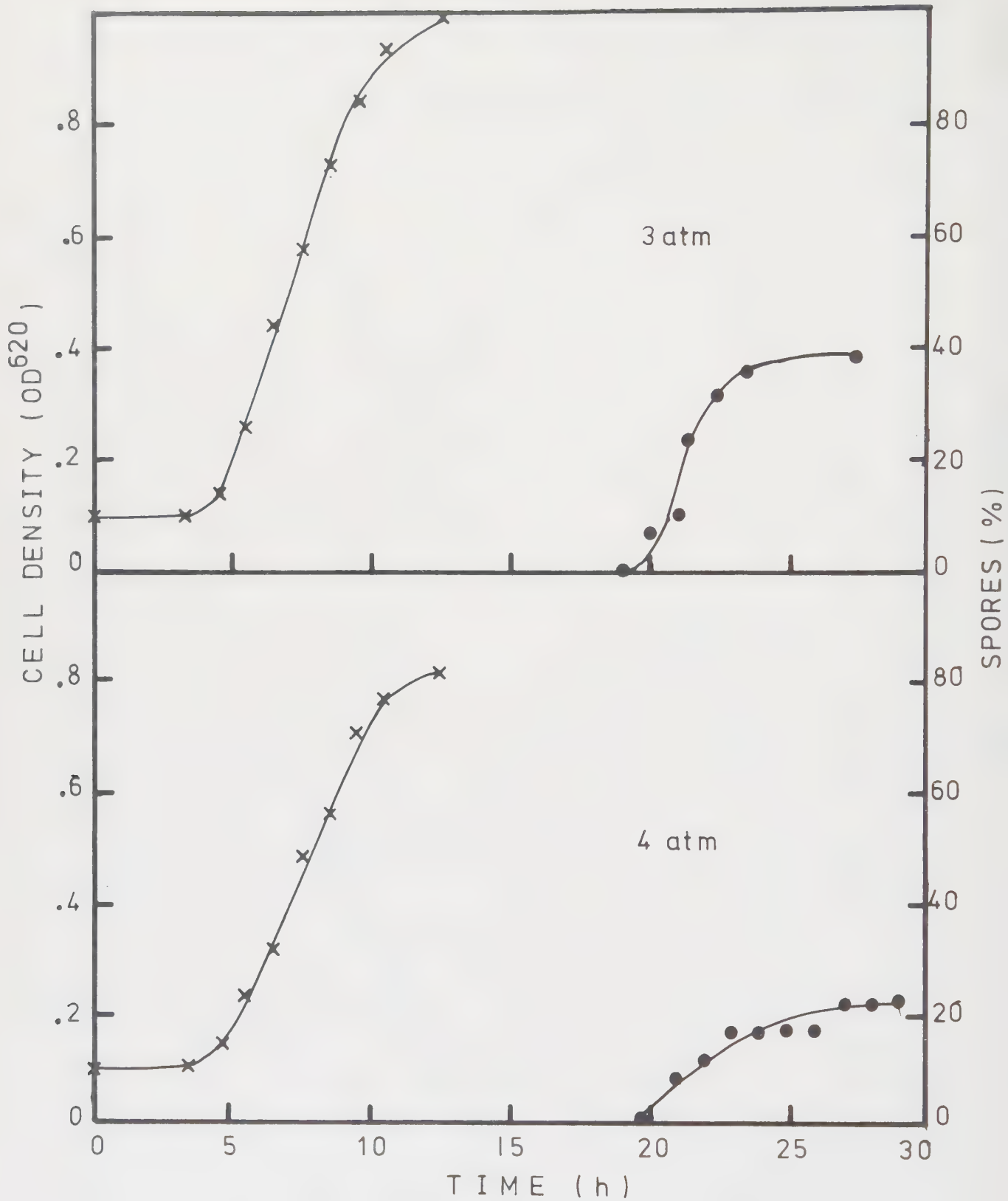


FIG 7. GROWTH (x) AND SPORULATION (•) OF B. CEREUS IN 3 AND 4 ATM OF AIR.

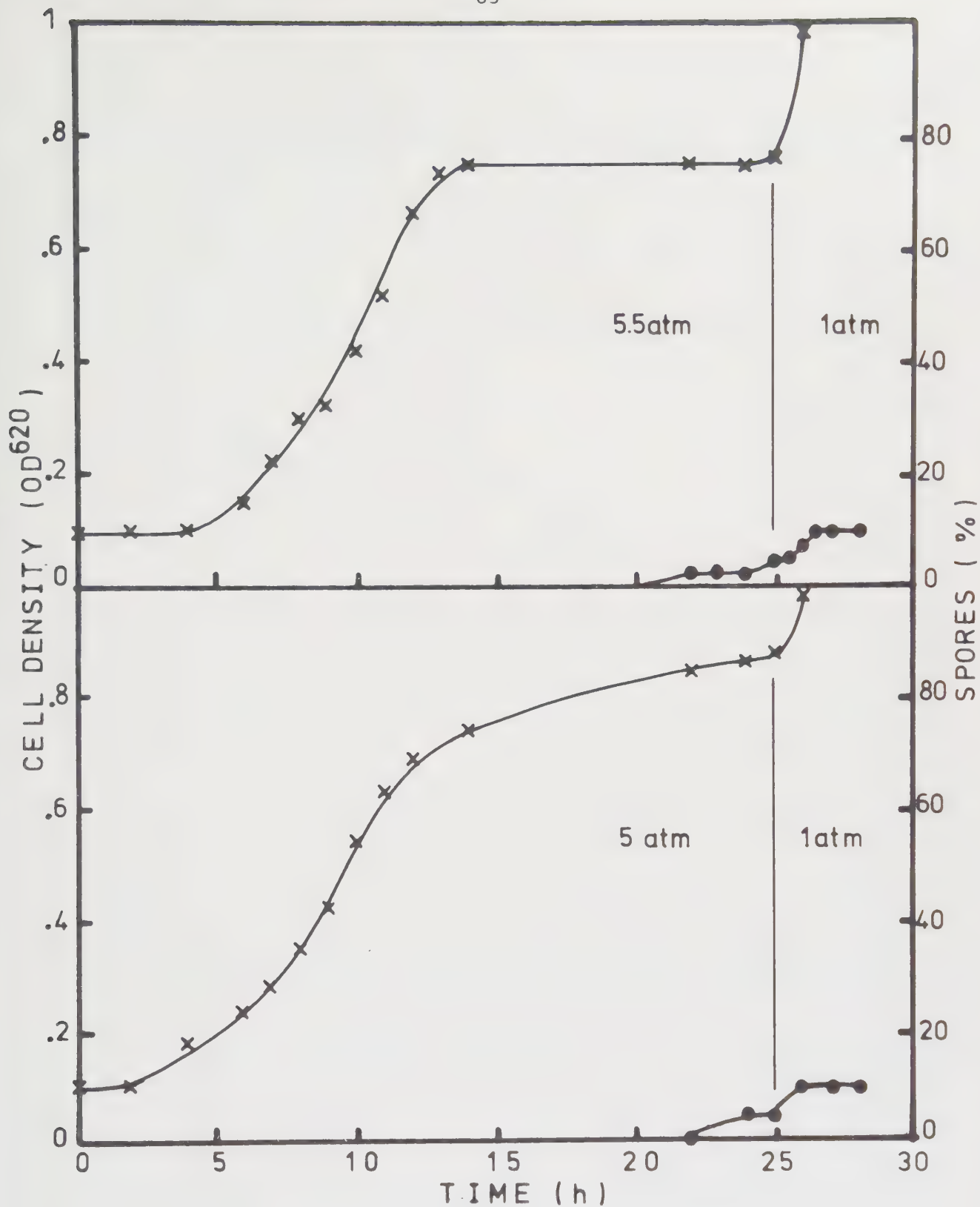


FIG 8. GROWTH (x) AND SPORULATION (•) OF *B. CEREUS* IN 5 AND 5.5 ATM OF AIR. AFTER 25 HOURS THE PRESSURE WAS RELEASED.

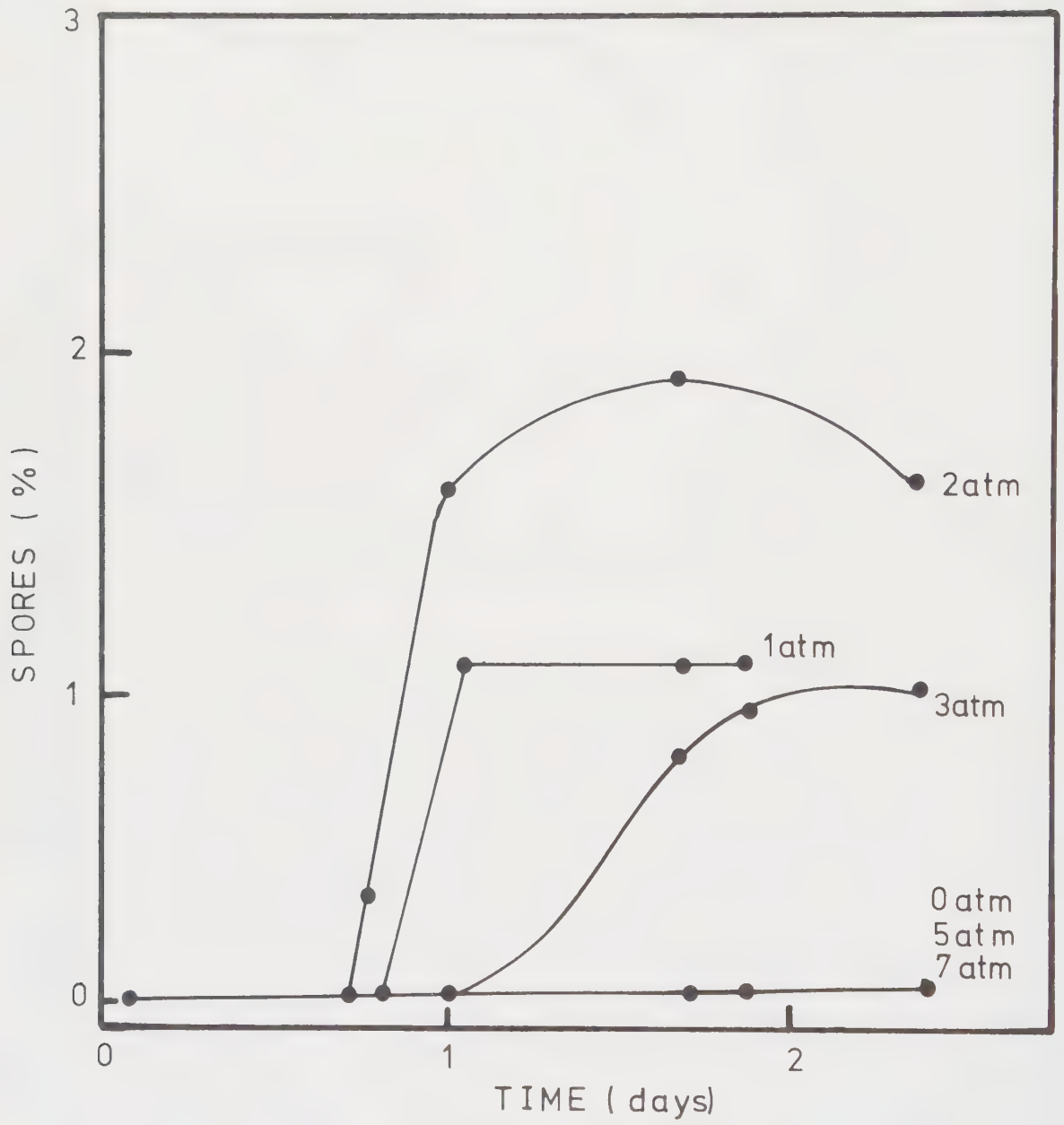


FIG 9. SPORULATION OF *B. STEAROTHERMOPHILUS* IN 0 - 7 ATM OF AIR.

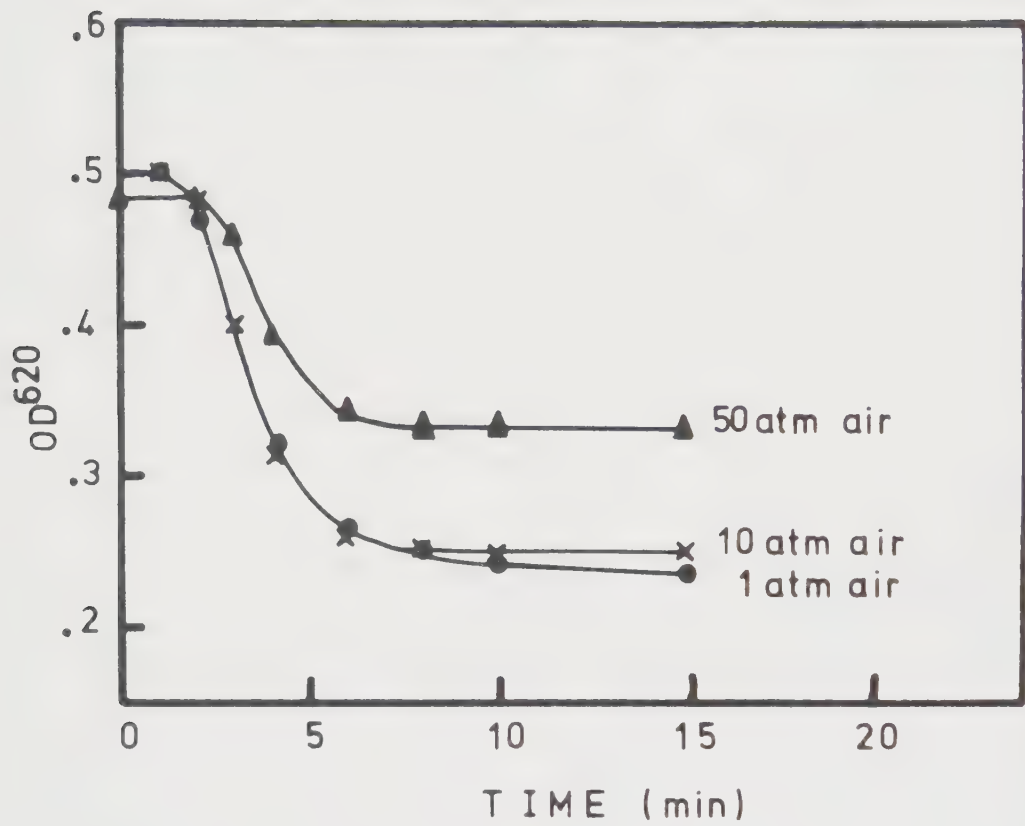


FIG 10. GERMINATION OF *B. cereus* SPORES IN 1, 10 AND 50 ATM OF AIR.

A HIGH PRESSURE FERMENTER

Summary

A high pressure fermenter has been developed. The capacity is 4 l culture and 15 atm gauge pressure. The principle of this design is that the pressure fermenter should produce all analyses and control facilities that are offered by a standard laboratory fermenter. The pressure fermenter offers extended possibilities in the research on effects of dissolved gases on microorganisms. It also provides a tool for increasing the gas transfer rate without increasing the mechanical stress on the organism. The pressure fermenter has been tested in a methanol-SCP process.

Introduction

There are two aspects behind the development of this high pressure fermenter. Firstly, in the previous discussion it appeared, that studies on the effects of high air pressure on microorganisms are difficult to evaluate if there is no analysis of dissolved oxygen. The reason being that the organisms, if aerobic, consume oxygen so that there will be a discrepancy between the dissolved oxygen concentration and the oxygen pressure in the fermenter, a discrepancy which will be unpredictable since it depends on growth rate, cell density, yield coefficient for oxygen and oxygen transfer coefficient ($K_L a$). Furthermore it is always desirable to keep as many parameters as possible under control. This means that a perfect equipment for these studies ought to be furnished with all those control systems that are used in ordinary laboratory fermentations, viz. pH control, temperature control, variable stirrer speed, sampling and feeding facilities and so on.

The second aspect behind this fermenter is the effect of high air pressure on the gas transfer. The oxygen transfer rate is decided by $\frac{dC}{dt} = K_L a \cdot (C_e - C)$ where $K_L a$ is the volumetric oxygen transfer coefficient and C and C_e are the actual dissolved oxygen concentration and the dissolved oxygen concentration in equilibrium with the gas phase. Thus, the oxygen transfer rate can be increased either by increasing $K_L a$ or by increasing C_e . The former is mainly achieved by increasing the power input or increasing the

the superficial air velocity. The design of the equipment in the fermenter influences greatly the $K_L a$ -value and these circumstances have been extensively studied (204, 203). A recent review on the $K_L a$ in fermentation media is given in a publication by Robinson and Wilke (205).

The other possibility to increase the oxygen transfer rate, that of increasing the equilibrium concentration of oxygen, is in practice met only in two ways: by increasing the percentage of oxygen in the inlet gas or by increasing the total air pressure in the fermenter. Both methods yield increased partial pressure of oxygen which increases the solubility according to Henry's law (185). In this utilization of the pressure fermenter the pressure is applied in order to keep the dissolved oxygen concentration above a critical value.

There are few investigations reported concerning the use of increased pressure in order to increase the oxygen transfer rate. Elsworth et. al. have studied the effect of pressure on the sulfite oxidation rate in a fermenter (206) and on growth of Aerobacter cloacae (207). This organism grew logarithmically to a certain concentration and then linearly which was supposed to depend on the onset of oxygen limitation. The cell density at which this occurred increased when the air pressure was increased and the productivity during the subsequent linear growth also increased with increasing pressure. These experiments were performed at 1.68 atm and 2.36 atm total pressure. Also in gluconic acid fermentation increased air pressure yielded higher productivity (208). However, Candida lipolytica grown on hexadecane was shown to be inhibited as soon as the air pressure was increased (209).

None of the referred investigations included dissolved oxygen measurements and all of them were performed with the high air pressures from the very beginning of the process. If the purpose of the application of high pressure is to extend the logarithmic growth phase and/or to increase the productivity during the oxygen limitation phase then it is enough to compress when the dissolved oxygen concentration approaches the critical level. Furthermore the toxic effect of oxygen makes it risky to use too high an oxygen pressure early on in a process when the consumption rate is low.

There are some other aspects on the use of high air pressure than just the effects on oxygen concentration and oxygen transfer rate. This technique will also yield increased concentrations of dissolved carbon dioxide and nitrogen and a hydrostatic pressure. The result of the literature review of this thesis makes it less probable, but not impossible, that the hydrostatic pressure will influence the process as long as the pressure is below 10 atm. However the increased nitrogen and carbon dioxide concentrations might well influence the process.

Some designs of laboratory pressure fermenters have been published earlier. Wayman and Wein described a 7 l pressure fermenter for 13.4 atm gauge pressure which they used for cultivation of Candida lipolytica (209). It was designed as an ordinary laboratory fermenter but was made solely of stainless steel. Brown and Barrett (210) made another approach. They built an outer pressure resistant (6 atm) shell around an ordinary 14 l laboratory fermenter and furnished the bottom and lid with connectors which admitted the use of the ordinary thermostating and sampling systems. During pressure operation the stirrer was substituted by a ring sparger. Safety problems connected with the use of pure oxygen under pressure was also discussed in their work.

However none of these systems provided any method of using electrodes and no discussion was made concerning additions to the fermenter during the process.

The purpose of this work was to develop a laboratory fermenter for high pressure cultivations which was not restricted in the normal control functions of a laboratory fermenter. That means that it should offer facilities for pH, foam- and dissolved oxygen control and measurements with other electrodes which can stand the pressure. It must furthermore admit adaptation to continuous cultivations.

In order to test the equipment it has been used in a methanol-SCP process.

The pressure fermenter.

The main principle of the pressure fermenter is shown in fig. 1. Fig. 2 is a photograph of the pressure fermenter. Letters in brackets refer to details in figure 1. Some of these details are marked also in fig. 2. The fermenter consists of a bottom part, the fermenter body (a), which is furnished with a magnetically (b) connected stirrer and baffles (c). The total volume of the fermenter body is 5.1 litres. Close to the bottom are four connections with valves for sampling (d), air inlet (e) and continuous medium flow (not shown in fig. 1). The fermenter body is sealed by an inner lid (f) which is furnished with 9 holes for standard Chemoferm-connectors. This lid is not pressure resistant and it finishes the sterile part of the fermenter. The fermenter body with connecting electrodes, condenser and tubings for additions can be autoclaved. After this the inner lid is furnished with a stative (see fig 2, not shown in fig. 1) into which at least two peristaltic pumps with variable speed (g) can be fixed. Each pump is connected to a 220 ml glass cylinder (h). With some effort a 3rd pump and a smaller reservoir, eg. for antifoam, may also be built in. The mounting is finished by the application of the pressure top (i) which is placed over the stative (a hoist is used for this operation).

The wires from the electrodes and pumps are taken out through two holes (j) in the top and then connected to the electrical pressure tight connectors which are shown in fig. 3. Each of the two connectors are furnished with 5 subminiature coaxial contacts which makes it possible to transfer 20 signals through the pressure top.

The outlet air tube is then connected to a cross tube (k) provided with a pressure release valve, a pressure gauge and an outlet air valve. The outlet air valve is furnished with an air heater (l) and a filter (m) which is autoclaved with the connecting tube to the condenser.

The complete equipment is placed on a motorstand (Chemoferm 10 L) which offers possibilities for stirring speed from zero to 3000 RPM. The fermenter is cooled by a flow of water through a cooling finger (n) mounted in one of the connectors of the inner lid. The water is transferred through the pressure top and flows from the cooling finger through the condenser and out through the pressure top again (o). Heating is provided by an electrical heating band (p).

The complete equipment is designed for operation at pressures up to 15 atm gauge pressure.

When the pressure is raised the air partially passes through the filter (q). This flow will stop when the pressure in the top is equal to the pressure in the fermenter body. Then all air will flow directly from the fermenter body to the outlet air valve, an arrangement which is necessary in order to prevent abundant condensation inside the pressure top and to prevent too long residence time of the outlet air which would cause delayed responses in the gas analyses.

Instrumentation.

In principle any electrode that can stand the pressure may be mounted in the inner lid and the signals are transferred to or from the electronics outside the fermenter via the pressure tight electrical connectors shown in fig. 3.

Foam control is achieved either by dosing of antifoam from a built in reservoir, controlled by a conductivity electrode, or by using a mechanical foambreaker mounted through the center connector (all connectors in the lid are fully interchangeable). A disc may also be mounted on the top of the stirrer.

For temperature control a thermistor is mounted through a connector in the inner lid. The signal is fed through the electrical connector in the pressure top to a temperature controller that controls the heating and/or cooling system.

Dissolved oxygen electrodes of the galvanic type described by Johnson et. al. (211) have been used in the pressure fermenter at pressures up to 6 atm. The response of the electrode to the increased air pressure is shown in fig. 4.

It is evident that the response was not linear with increasing air pressure. Johnson et. al. (211) reported a linear response of their electrode at oxy-

gen pressure up to 0.21 atm. In order to check the non-linearity of the curve was depending on the total gas pressure a corresponding calibration curve was made with a mixture of nitrogen and oxygen at one atm. Fig. 4 shows that the unlinearity is not depending on the total gas pressure, but rather is a characteristic of the electrode at high oxygen partial pressure. It means that the DO-signal during a pressure fermentation can be used without corrections as far as the signal is below the one corresponding to 1 atm of air (100% air sat.).

pH measurements at increased pressure offer problems. At pressures up to 5 atm gauge pressure a standard electrode (eg. Radiometer GK 4033 C) can be used. There are systems on the market that can operate at pressures up to 25 atm but these are industrial systems which require a large amount of space.

An antimon electrode (Radiometer P 301) was applied in an attempt to find a pressure resistant pH controlling system. Generally the antimon electrodes are not as stable and especially not as reversible as glass electrodes, but in a system where mainly a control of constant pH is needed it was thought that this might be an approach. However this electrode, with a calomel electrode as a reference, proved to be very sensitive to dissolved oxygen concentration. This fact makes it impossible to use the antimon electrode in fermentation. The sensitivity to oxygen was -0.8 pH units per 0.2 atm increase of oxygen partial pressure in 67 mM phosphate buffer at pH 7 and 25°C.

The main reason why an ordinary pH electrode cannot be used under pressure is that the glass bulb constitutes a closed system that will implode at too high pressure. Furthermore, one has to make sure that there is a slight over pressure inside the reference electrode so that the normal flow of electrolyte through the ceramic plug is not hindered. However, there are possibilities to manipulate the glass electrode for operation under high pressure. In principle, the electrode is opened and resealed by a compressible seal. Some designs, according to this principle, are published (212, 213, 214).

The reservoir for additions are built into the pressure top in order to make it possible to use simple peristaltic pumps for dosing of antifoam, pH control, nutrient feeding and so on. This construction makes it necessary to include a method for measuring the levels in the reservoirs or the amounts of dosed liquid, since visual reading cannot be made. A dose monitor (DM 1, Servo Chem AB), which continuously records the accumulated amount of dosed liquid is used for this purpose. The principle of this method has been reported elsewhere (215).

When the pressure fermenter is to be used in a chemostat process a level sensing conductivity sensor is mounted in the inner lid. The signal is fed to the controller outside the pressure fermenter which controls the pumps (or pump and valve) for medium flow through two of the four valves close to the bottom of the fermenter body. A pump which can operate against the pressure is then needed for the input medium flow.

Cultivation of a methanol oxidizing bacteria in the pressure fermenter.

Instrumentation. For the cultivation of methanol oxidizing bacteria the outlet air was fed to a flow meter, an alcohol controller (Alcotrol Ac 3, ServoChem AB), a carbon dioxide infrared analyser (Lira 300, MSA Ltd) and a gas chromatograph (Varian 920) fitted with a molecular sieve column for intermittent oxygen analysis. The pH electrode was connected to a pH controller (Metrohm E 512 with Impulsomat E 472) and the dissolved oxygen electrode was connected via an I-K potentiometer to the multipoint recorder which was used to record all the continuously measured parameters.

The pH was kept at 6.0 and the methanol concentration at 0.35% (v/v) by additions of ammonia and methanol respectively. The amounts of dosed liquids were continuously recorded by two dose monitors (DM1, ServoChem AB).

Inoculum. Methylobacterium methanolicum, grown in a methanol limited chemostat, was used to inoculate 3.5 l medium to the initial cell density 2 g/l.

Medium. A mineral salts medium with ammonia as nitrogen source and with 0.35% methanol was used. The medium was composed to yield about 15 grams biomass per litre.

Aeration. The stirrer was run at 1 200 RPM and no baffles were used. The air flow was constant at 3.5 l/min throughout the process.

The temperature was kept at $30 \pm 0.1^{\circ}\text{C}$.

Results and legend to fig. 5.

A copy of the recorder signals from a cultivation is shown in fig. 5. The record has been completed with the data from the oxygen analysis. The following markings and scales are used in fig. 5: Dissolved oxygen (DO) is given in percentage of corresponding value at equilibrium with one atm of air, scale 0 - 100%. Oxygen concentration in outlet air (O_2), scale 0 - 20%. Methanol concentration (m), scale 0 - $\sim 0.5P\%$, where P is the total gas pressure. Carbon dioxide concentration in outlet air (CO_2), scale 0 - 6%. pH (pH), scale 7 - 2. Amount of dosed methanol (D_C), scale 0 - 210 ml of 100% methanol. Amount of dosed ammonia (D_N). Scale 0 - 150 ml of a water solution with 4.85 g NH_3 /l.

Discussion on fig. 5.

As seen from fig. 5 the methanol signal decreases when the pressure is increased. The partial pressure of methanol in the fermenter is a function of the concentration in the fermenter liquid and it is independent of the total gas pressure. Therefore the concentration of methanol in air will be depending on the total gas pressure. The response of the methanol analyser to concentration of methanol in the air is linear within a large range. Thus, the methanol signal ought to be proportional to the reciprocal of the gas pressure. This hypothesis was tested in the experiment shown in fig. 6, which shows that there is a discrepancy between the predicted and the measured value. However, this discrepancy seems to stabilize after the first compression.

The dips in carbon dioxide concentration caused by compression may initially be explained in a similar way. When the amount of air is increased within a short time the concentration of carbon dioxide in the air will be reduced but, contrary to the case of methanol, the carbon dioxide is continuously produced and the concentration in the air at equilibrium is de-

pendent on this production rate and air flow. If these parameters are not changed after the compression the concentration of carbon dioxide should return to the initial one. This was verified in fig. 5. As a consequence of this the partial pressure of carbon dioxide and also the dissolved carbon dioxide concentration will increase in proportion to the total gas pressure.

The dissolved oxygen signal increased at the compressions as was expected from the previous discussion. When the DO-signal does not change quickly, the oxygen consumption rate is approximately equal to the oxygen transfer rate. This situation can be written for two air pressures P_1 and P_2 :

$$OCR_1 \sim OTR_1 = K_L a (100 P_1 - C_1) \quad (\text{eq 1})$$

$$OCR_2 \sim OTR_2 = K_L a (100 P_2 - C_2) \quad (\text{eq 2})$$

If the oxygen consumption rate is not changed and if $K_L a$ is independent of the gas pressure then the increase in dissolved oxygen concentration can be calculated from eqs 1 and 2:

$$C_2 = 100 (P_2 - P_1) + C_1 \quad (\text{eq 3})$$

In these equations the dissolved oxygen concentration in equilibrium with one atm of air is settled to 100%.

When eq 3 was applied to the data of fig. 5 the observed values of dissolved oxygen were 19 - 24% lower than the predicted one in all cases except the first compression in which the observed value was 28% higher than the calculated one.

At arrow A in fig. 5 more mineral salts were added to the fermenter since the observed decrease in growth rate, measured as slower production of carbon dioxide and slower consumption of ammonia and oxygen, was caused by mineral salts starvation. The dry weight was then 14.4 g/l. The methanol dosing was also switched off at arrow A and at arrow B methanol starvation began. Methanol dosing was then restarted.

The pressure fermenter as a research tool

No efforts were made to evaluate the process parameters during this cultivation, since the purpose was only to demonstrate the function of the pressure fermenter. However, some interesting aspects can be mentioned. The pressure fermenter provides a method for increasing the gas transfer rate in a microbial process without causing additional mechanical stress. Furthermore, it offers possibilities to study some of the effects that will occur deep in a very high fermenter. A 40 meter high air lift fermenter will have a gas pressure of 5 atm close to the bottom. It also provides means for studies of the effects of high oxygen concentration on microbial processes under controlled conditions. A great part of the literature on effects of high oxygen pressure on microorganisms that was referred to in the literature review suffered from the drawback that too many process parameters were unknown. The complete control system also admit such studies on special processes or conditions which cannot be achieved in uncontrolled cultivations in test tubes or shaking flasks.

Another very interesting parameter that conveniently can be studied with the pressure fermenter is the dissolved carbon dioxide concentration, the effect of which will now be further discussed in spore germination studies. It seems as if this gas has a depressing effect on some microbial reactions if the concentration is increased. This offers interesting aspects on preservation. Actually, this gas is prevalent in many foods either naturally, as in beer, or artificially as in some packages into which gases are sometimes added in order to give a protective atmosphere for the foodstuff. However, the knowledge about how carbon dioxide at high partial pressures influences life reactions is very limited.

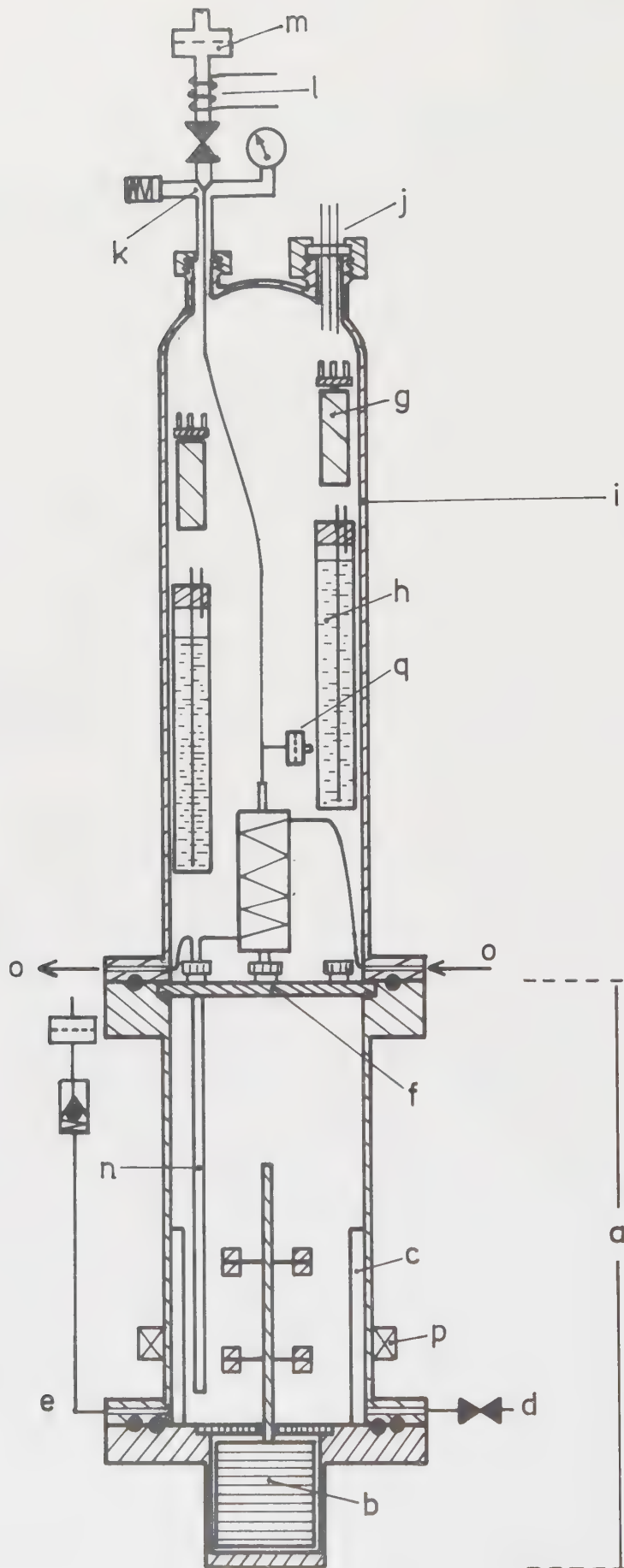


FIG 1. THE PRINCIPLE OF THE HIGH PRESSURE FERMENTER. THE LETTERS REFER TO THE DESCRIPTION IN TEXT.

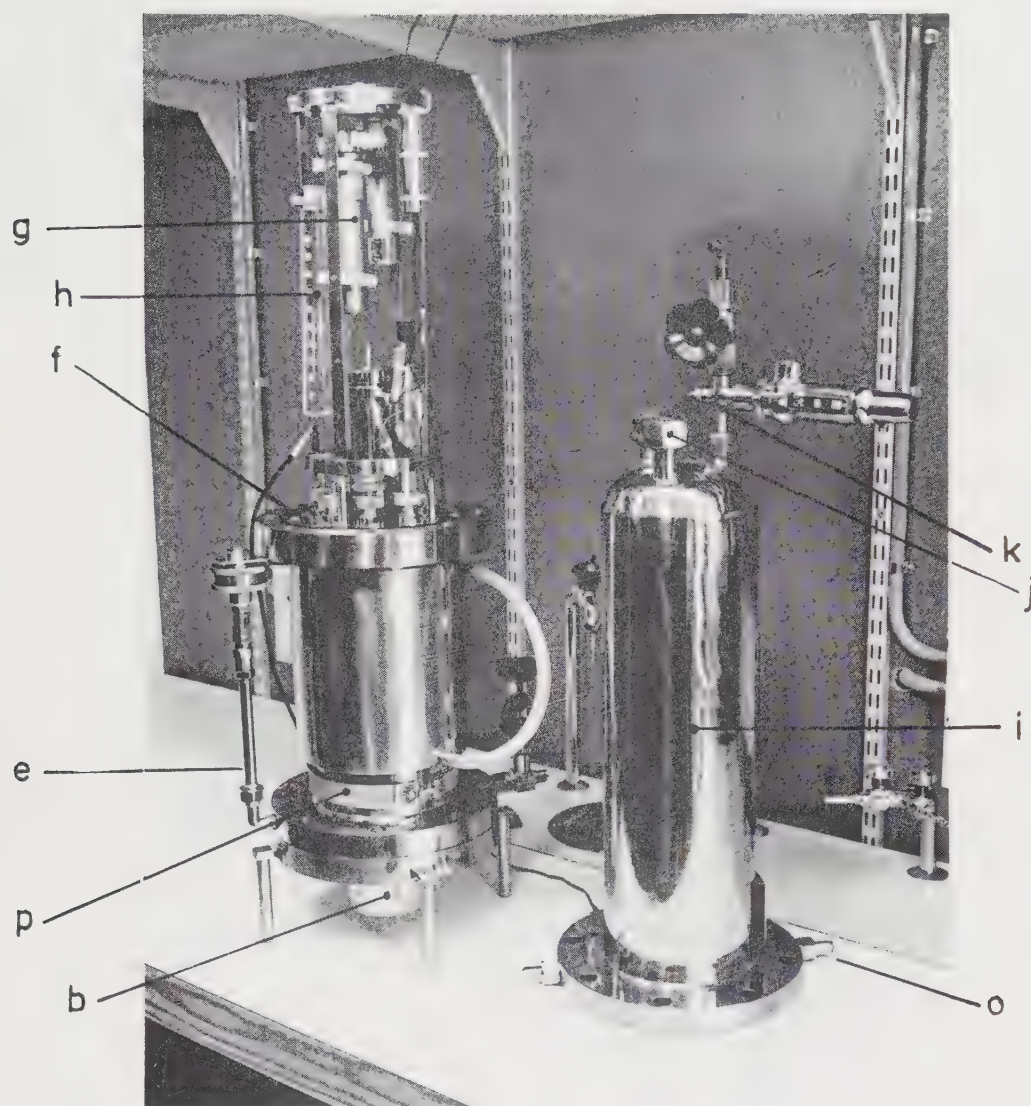


FIG 2. THE HIGH PRESSURE FERMENTER WITH DEMOUNTED TOP.
THE LETTERS REFER TO DETAILES DESCRIBED IN TEXT.

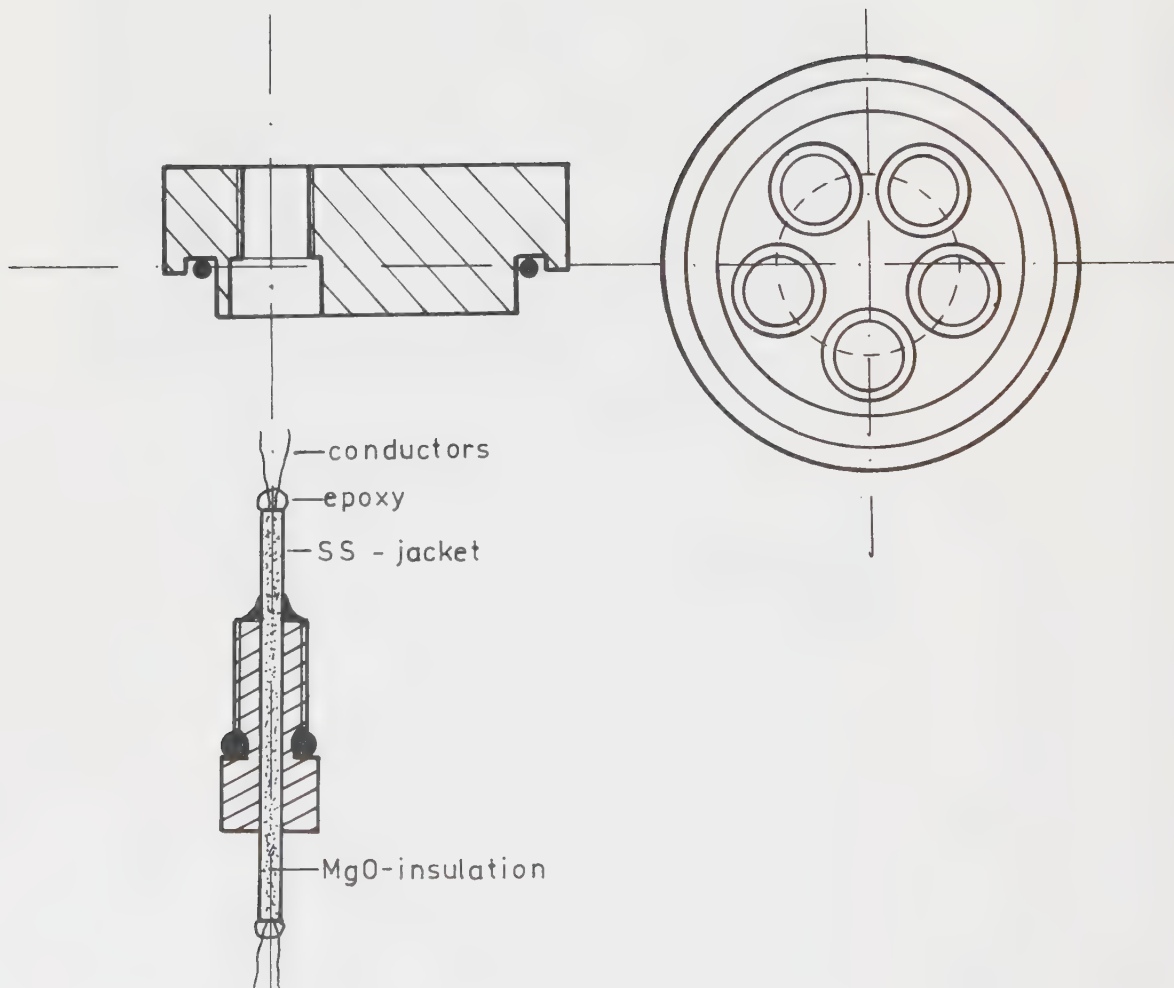
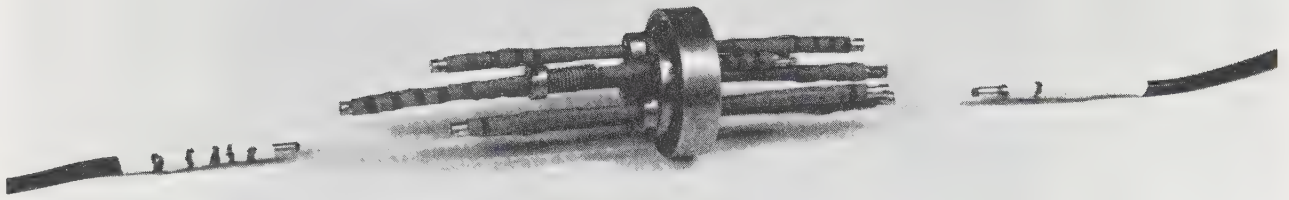


FIG 3. PRESSURE TIGHT ELECTRICAL CONNECTORS. THE STAINLESS STEEL JACKETED THERMOELEMENT (HONEYWELL AEROPAK 12-30-20-13) IS WELDED INTO THE STEEL SCREW AND THE MAGNESIA OXIDE INSULATION IS SEALED AT BOTH ENDS BY EPOXYRESIN. THE ELECTRICAL WIRES ARE CONNECTED TO SUBMINIATURE COAXIAL CONTACTS (BURNDY RCDX K-1), ISOLATION RESISTANCE $> 100 \text{ M}\Omega$, CONTACT RESISTANCE $\sim 1\Omega$.

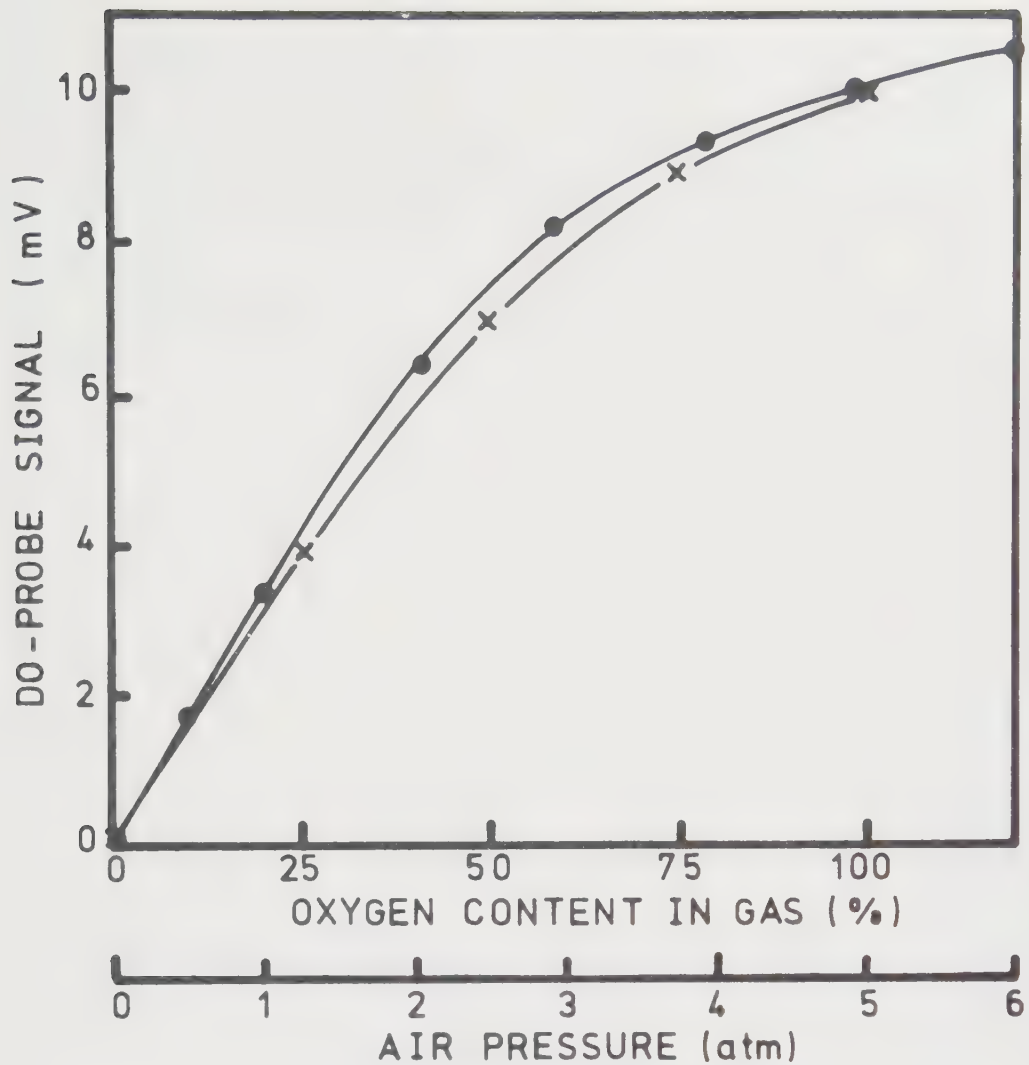


FIG 4. THE RESPONSE OF THE DO-PROBE TO INCREASED PARTIAL PRESSURE OF OXYGEN, WHICH WAS ACHIEVED IN TWO WAYS. THE UPPER CURVE SHOWS THE RESPONSE WHEN THE TOTAL AIR PRESSURE WAS INCREASED, AND THE LOWER CURVE SHOWS THE RESPONSE WHEN THE OXYGEN CONTENT IN A NITROGEN-OXYGEN MIXTURE AT 1 ATM WAS INCREASED.

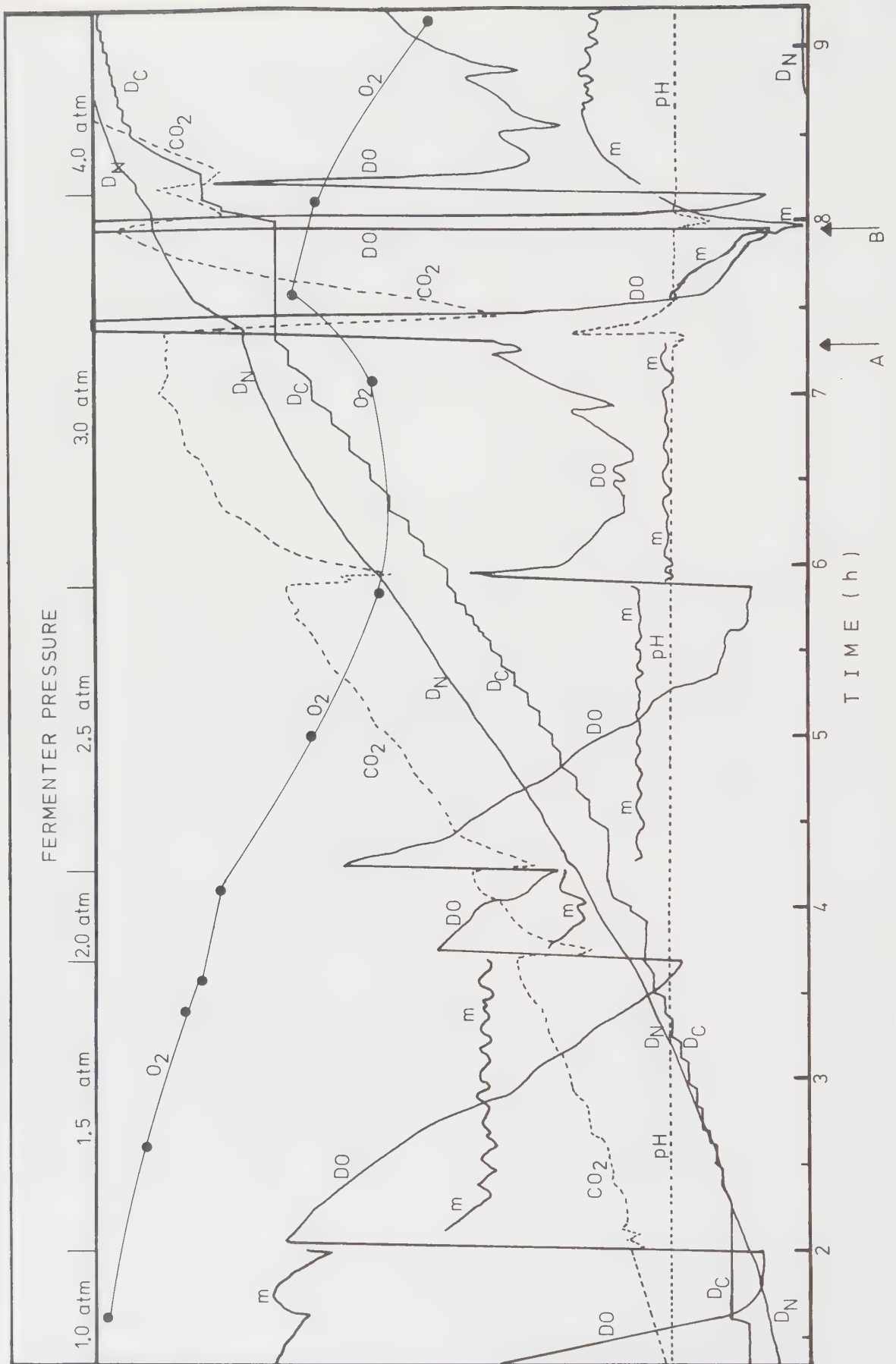


Fig. 5 Legend on page 93.

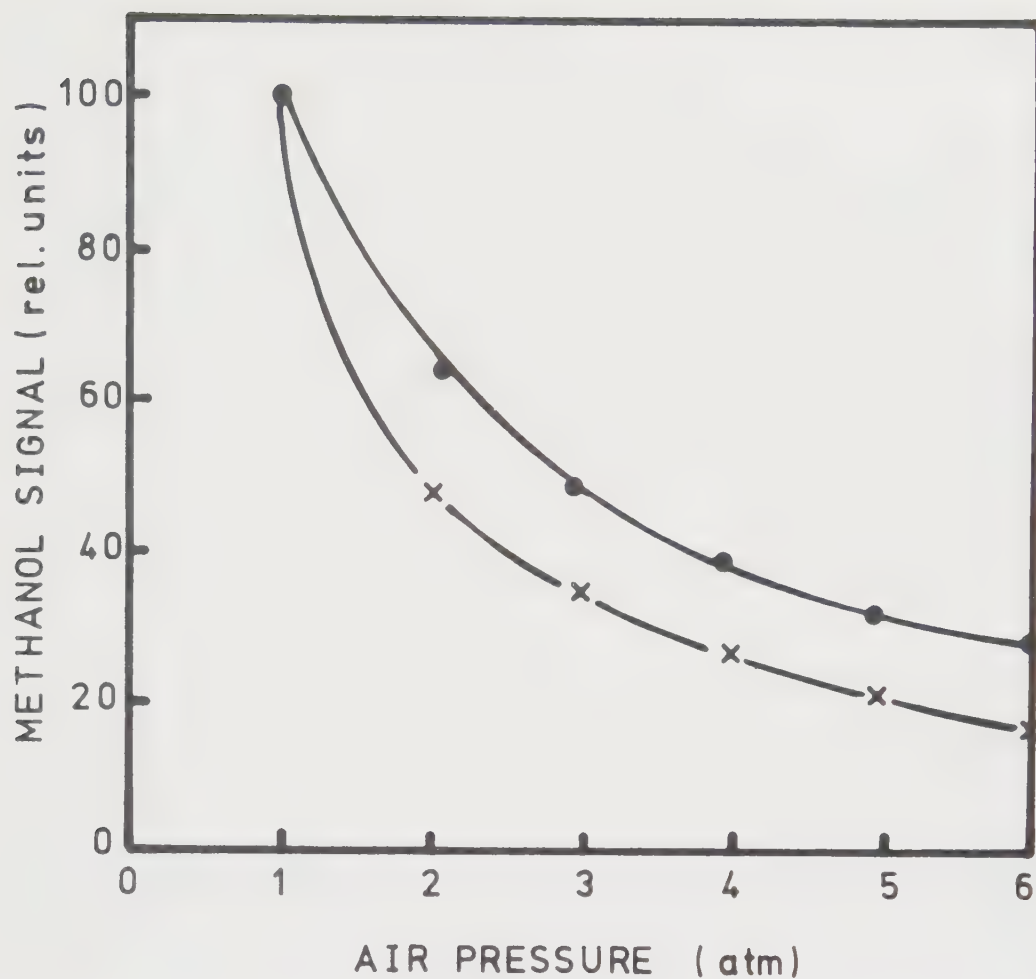


FIG 6. THE RESPONSE OF THE METHANOL-ANALYZER (ALCOTROL AC 3) TO INCREASED AIR PRESSURE. THE UPPER CURVE SHOWS THE MEASURED VALUES AND THE LOWER CURVE SHOWS VALUES PREDICTED AS DESCRIBED IN TEXT.

THE GERMINATION OF BACILLUS CEREUS SPORES AFTER EXPOSURE TO HIGH GAS PRESSURE

Summary

The germination kinetics as measured by lag time, maximum germination rate and extent of germination, was investigated in spores of B. cereus which had been kept under 100 atm of helium or air or 50 atm of carbon dioxide. In no case was there any difference in the kinetics between these spores and the controls in atm of air. This means that these spores are very resistant to high oxygen pressure. No germination inducing capacity was noticed by these treatments.

Introduction

The mechanism of spore germination is still unknown although many relations are described or suggested. For a review on this topic see (216). One hypothesis concerning the early events during spore germination emphasizes the importance of ion transport or ionization within the spore as significant steps (217, 218, 219, 220). Furthermore the fact that high hydrostatic pressure is a potent initiator of germination (64, 65, 66, 67) strongly supports the view that ionization within the spore is involved in the germination.

There is no proof that the germination involves metabolic reactions though one hypothesis suggests the metabolism of the spore germinant to form dipicolinic acid (216). Evidences for enzymatic participation in germination have been published (221, 222, 223, 224). If enzymatic reactions are involved the germination might be very sensitive to high oxygen pressure since metabolism in general shows such sensitivity. The mechanism of the oxygen sensitivity is not clearly understood but interactions with enzyme function, viz. oxidation of sulfhydryl groups, has been suggested (113). Other theories rather consider a metabolic malfunction, viz. increased production of hydrogen peroxide (121) or superoxide radicals (106).

In the previous brief survey of high air pressure effects on growth, sporulation and spore germination it was found that 50 but not 10 atm of air

partly inhibited the spore germination of Bacillus cereus. This is a very high resistance to oxygen as compared to that of vegetative cells.

This investigation deals with the effect of prolonged exposure of spores to high gas pressure on the viability of the spores as revealed by their germination kinetics. If the spores are killed, become superdormant or will germinate more slowly or be activated by this treatment then this will be revealed by their growth kinetics when they are allowed to germinate under ordinary conditions after the exposure. In the next chapter effects of the gas exposure during the germination, that is effects on the germination process itself, will be investigated. The gases that have been chosen in this experiment are air, from which effects of the high oxygen pressure might be expected, helium, as a control from which only hydrostatic pressure effects or effects by the gas itself are likely to occur, and carbon dioxide, which might be able to interfere chemically and which is also of interest as a potential gas for food preservation.

Materials and methods.

Spores. Bacillus cereus (SIK No 216) was obtained from SIK, Göteborg. Spores were prepared in a shake flask culture prior to each experiment. The cultivation medium (G-medium) is given on page 68. The cultivation temperature was 30°C and the spores were harvested when free from sporangia after 3 days. The spore suspension was washed 3 times in 67 mM phosphate buffer (pH 7) and resuspended in the buffer to OD⁶²⁰ ~ 0.8.

Gases. Carbon dioxide was purchased from AB Kolsyrefabrikerna, Lidingö and air and helium were purchased from AB Aga, Lidingö.

Germination initiator. In order to initiate germination a solution L-alanine and adenosine in 67 mM phosphate buffer (pH 7.0) was added to the spore suspension. The final concentrations were 2 mM adenosine and 10 mM L-alanine.

Pressure treatment. 200 ml of the spore suspension was transferred to a thermostated (30°C) pressure vessel (500 ml volume) furnished with a mixer a sparger and valves for inlet gas, outlet gas and sampling (see fig. 1 on page 76). The gas was flushed through the vessel for a quarter of an hour in order to wash out the air and then the pressure was applied.

Measurement of germination. A control sample was kept incubated in 1 atm of air at 30°C. Samples were taken from the pressure vessel and the control immediately after compression, after 1 hour and after 24 hours of pressure treatment. These spore suspensions were furnished with germination initiator and placed in a thermostated (30°C) cuvette in a spectrophotometer and the OD⁶²⁰ was recorded.

Calculations. The germination is expressed in terms of the function $1 - \frac{OD_t}{OD_i}$ where OD_t and OD_i are the optical densities at time "t" and time zero respectively. The relative number of germinating spores, the extent of germination, is expressed as $1 - \frac{OD_f}{OD_i}$ where OD_f is the final optical density which was mostly reached within 30 minutes. The rate of germination is expressed as the slope of the function $1 - \frac{OD_t}{OD_i}$ against time. The lag time until onset of the OD-decrease was also used for comparison of the germination kinetics.

Results and discussion.

A control experiment was performed in order to check if the initial concentration of spores in the suspension influenced the germination parameters calculated according to this method. The results are given in fig. 1 and they show that by the recalculation of the parameters the germination kinetics is properly described unless there are large differences in the spore concentration.

The germination of B. cereus spores after exposure to 1 and 100 atm of air during 24 hours and subsequent heat activation are shown in fig. 2. The corresponding germination of non activated spores is shown in fig. 3. These results do not reveal any evident differences in the germination pattern between pressure treated spore and their controls. The germination after 1 hour's exposure revealed the similar pattern.

Obviously a pressure as high as 21 atm of oxygen do not interfere with the properties of the spores with respect to their germination kinetics. This is an oxygen pressure which is deleterious to most living organisms (98) and that means that the list of resistance properties of this spore, and maybe also other spores, can be extended to include extreme resistance to high oxygen pressure. However, as was pointed out previously, the very

process of germination seems to be more susceptible to high air pressure since it was somewhat retarded by 50 atm of air.

The experiments with helium at 100 atm show quite the same results. The only effect one ought to expect from this experiment is the hydrostatic pressure effect. In these experiments no germination was initiated by pressure since the germination after pressure release was quite normal (fig. 2 and 4). Nor did the pressure activate the spores (fig. 3 and 5). The germination initiating effect of high hydrostatic pressure that was mentioned in the introduction was not shown until the pressure became in the order of 200 - 500 atm.

The curves shown in the figures originates from single experiments and in order to compare repeated experiments some kinetic parameters were chosen to describe the process. These are the lag phase until the first decrease in optical density was detectable (t_1), the germination rate $\frac{d(1 - \frac{OD_t}{OD_i})}{dt}$ calculated at the point of the curve where the rate was as highest, and the final decrease in optical density $1 - \frac{OD_f}{OD_i}$. These parameters from repeated experiments are listed in table I.

The results from the carbon dioxide experiments (fig. 6-7) inclined some effect on the germination kinetics. The pressure treated samples germinated more slowly than did the controls. The solubility of carbon dioxide in water under pressure is very high (15 ml/ml at 20 atm and 0°C) and there is one parameter besides the concentration that will be changed under these conditions - the pH. It was found that pH of the phosphate buffer (after aeration) had decreased to 6.2 when the samples were taken out from the pressure vessel. However, these pH changes were irreversible. The pH did not increase again during half an hour after the sampling.

In order to investigate what effect the pH might have on the germination kinetics, experiments were performed in which the relation between the salts in the buffer had been adjusted to give pH 6.2. The spores were germinated under ordinary conditions in these buffers and the results are shown in fig. 8 and table I.

From this it seems reasonable to assume that the small effect of carbon dioxide at 50 atm that was found above originated from the fact that the pH was irreversibly changed during the pressure treatment.

TABLE I.

ATMOSPHERE	LAG TIME	EXTENT OF GERMINATION $1 - \frac{OD_f}{OD_i}$	RATE OF GERMINATION $\frac{d \left(1 - \frac{OD_t}{OD_i} \right)}{dt}$
100 ATM AIR	90	100	152
	100	100	97
	100	96	80
100 ATM He	100	98	100
	100	102	78
50 ATM CO ₂	200	86	37
	100	95	55
	150	85	51
pH 6.2	120	93	45

THE GERMINATION PARAMETERS ARE EXPRESSED AS PERCENTAGE OF CORRESPONDING VALUE OF THE CONTROL. IN THOSE CASES WHERE THE GERMINATION WAS NOT FINISHED WITHIN 40 MIN, THE VALUE FOR EXTENT OF GERMINATION WAS CALCULATED FROM THE OD VALUES AT 40 MIN. ALL SAMPLES WERE TREATED WITH THE ATMOSPHERE (OR pH) DURING 24 HOURS WHEREAFTER THE SPORES WERE HEAT ACTIVATED AND FURNISHED WITH GERMINATION INITIATORS.

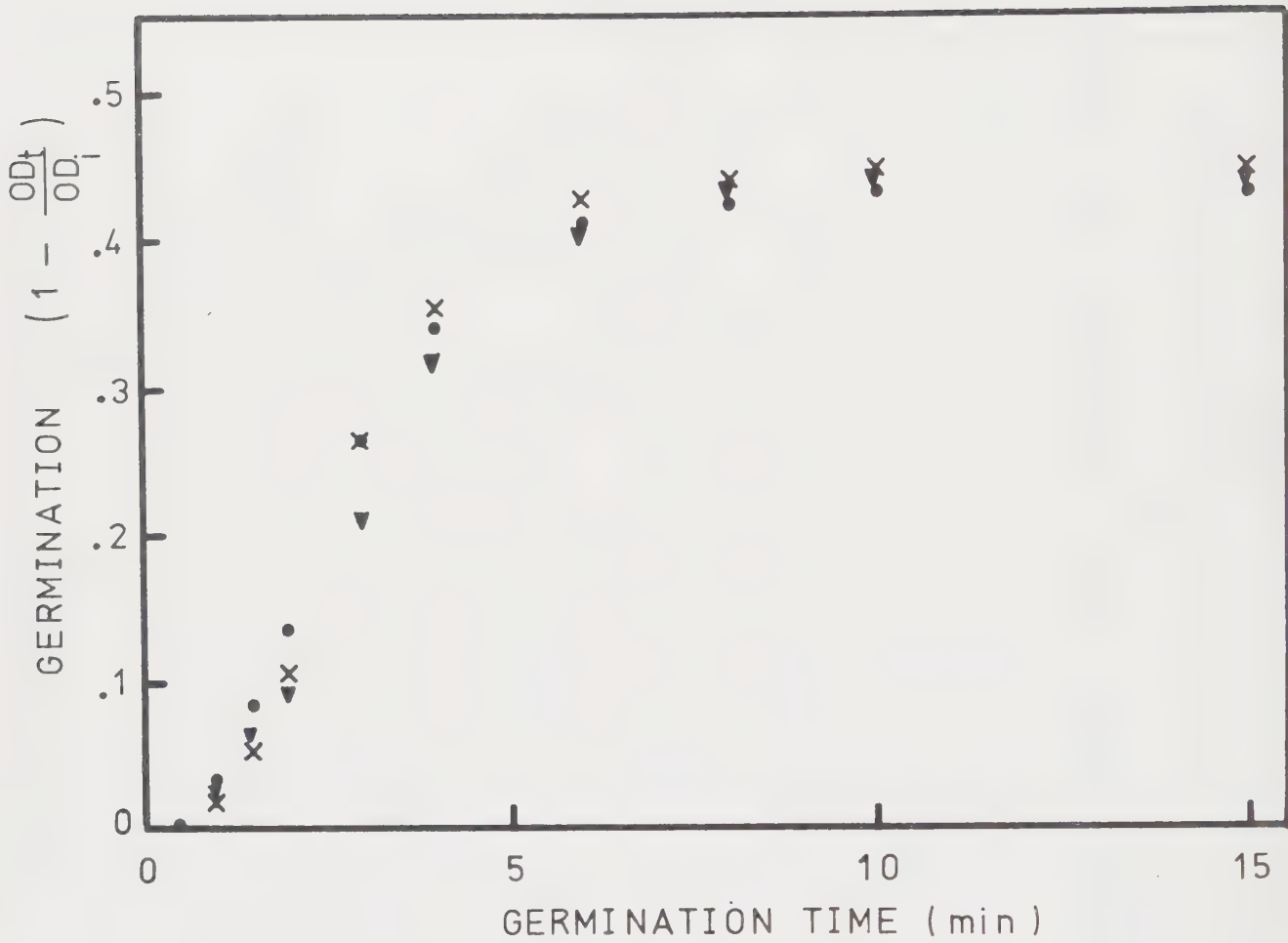


FIG 1. EFFECTS OF THE INITIAL OPTICAL DENSITY OF THE SPORE SUSPENSION ON THE FUNCTION $(1 - OD_t / OD_i) = f(t)$, THE INITIAL OPTICAL DENSITIES WERE 0.600 (•), 0.452 (x) AND 0.144 (▼).

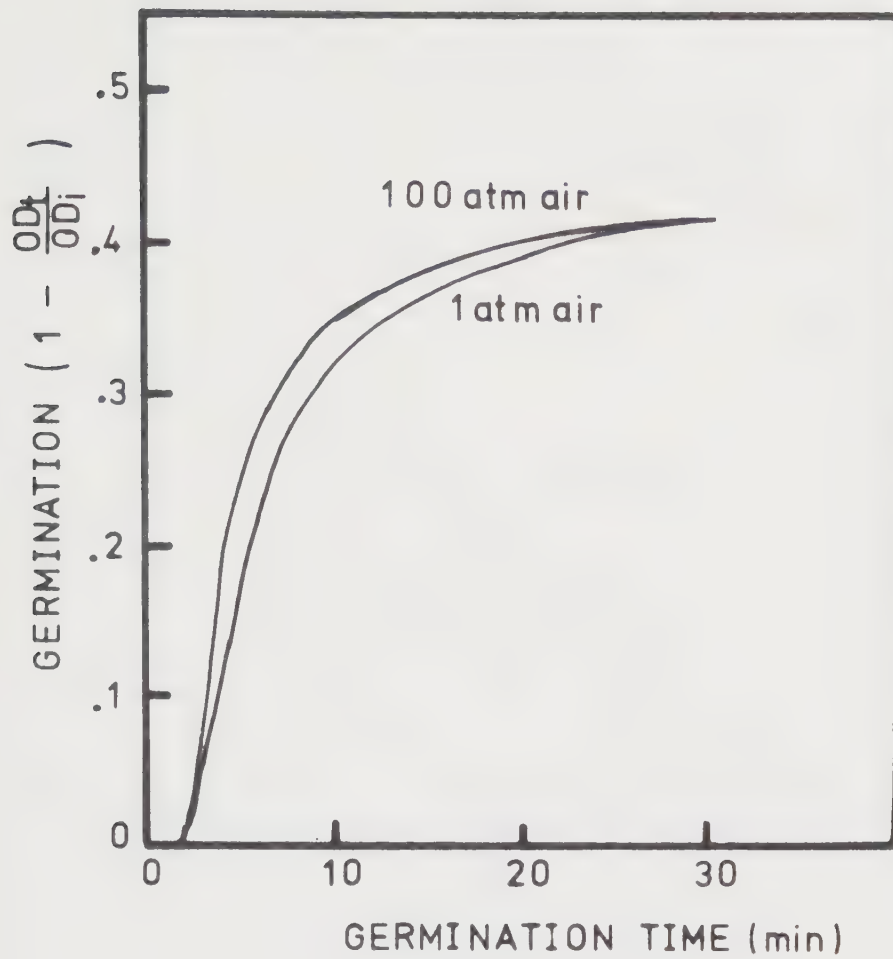


FIG 2. GERMINATION OF B. CEREUS SPORES AFTER INCUBATION IN 1 ATM AND 100 ATM OF AIR DURING 24 HOURS AND SUBSEQUENT HEAT ACTIVATION.

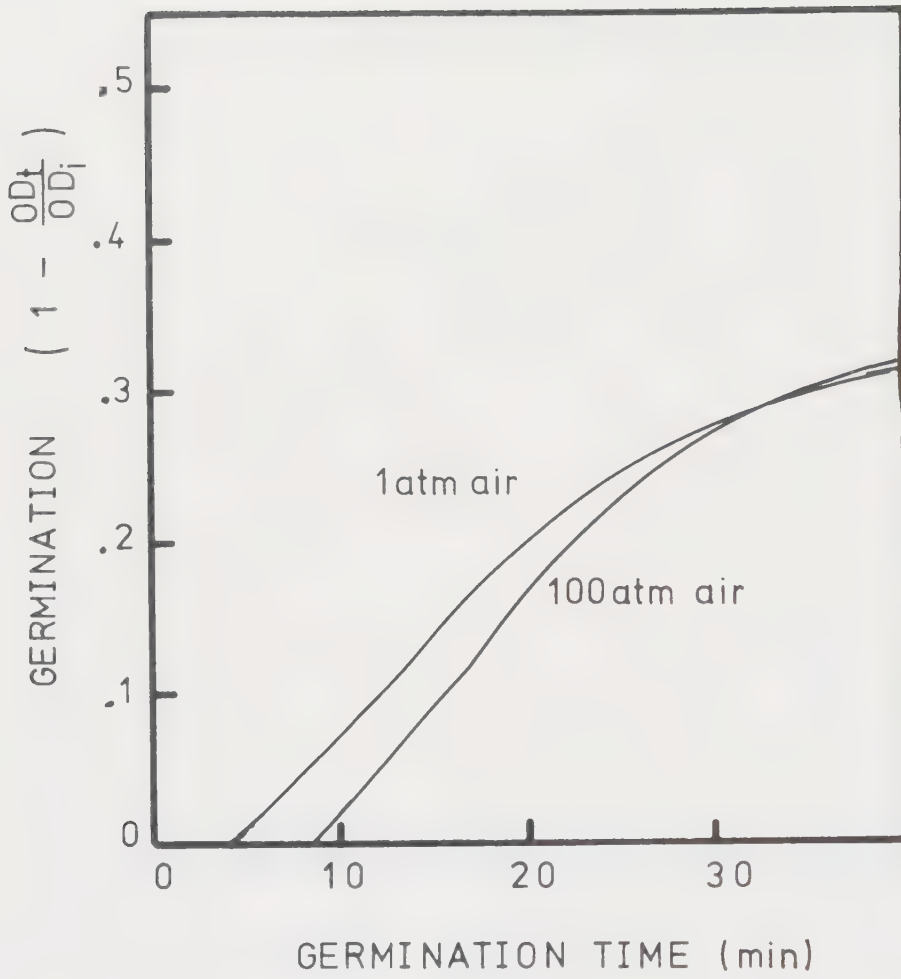


FIG 3. GERMINATION OF NON-ACTIVATED SPORES OF B. CEREUS AFTER EXPOSURE TO 1 ATM AND 100 ATM OF AIR DURING 24 HOURS.

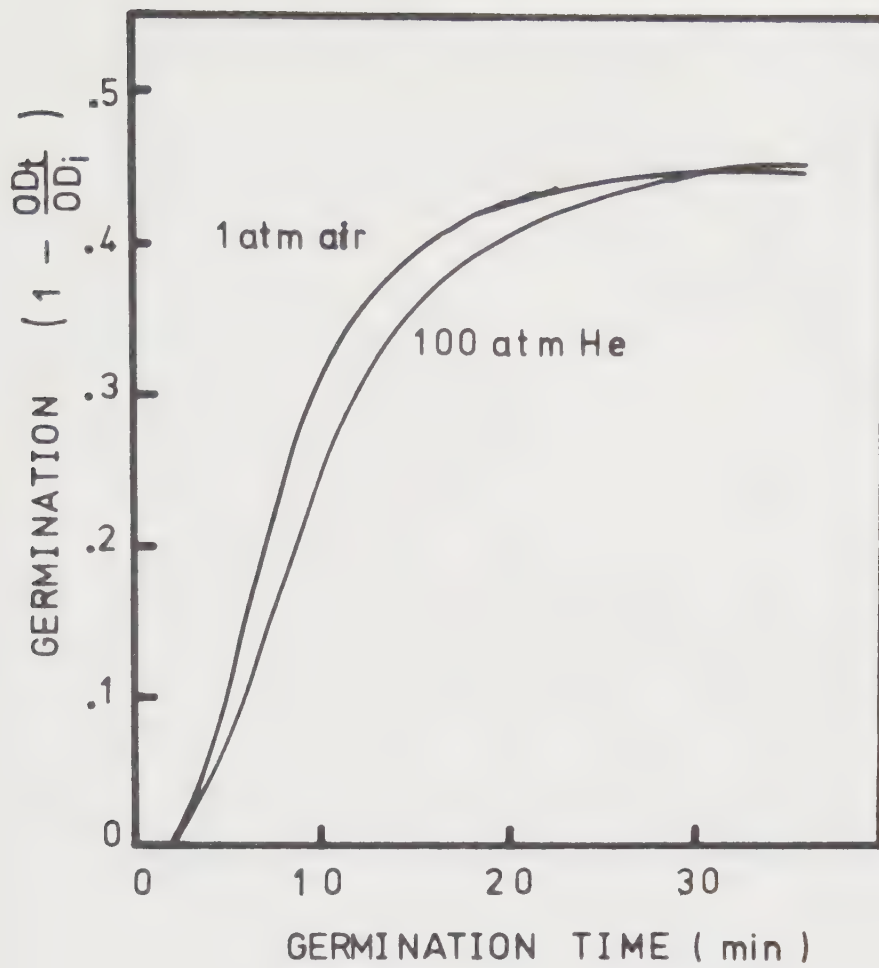


FIG 4. GERMINATION OF B. CEREUS SPORES AFTER INCUBATION IN 1 ATM OF AIR AND 100 ATM OF HELIUM DURING 24 HOURS AND SUBSEQUENT HEATACTIVATION.

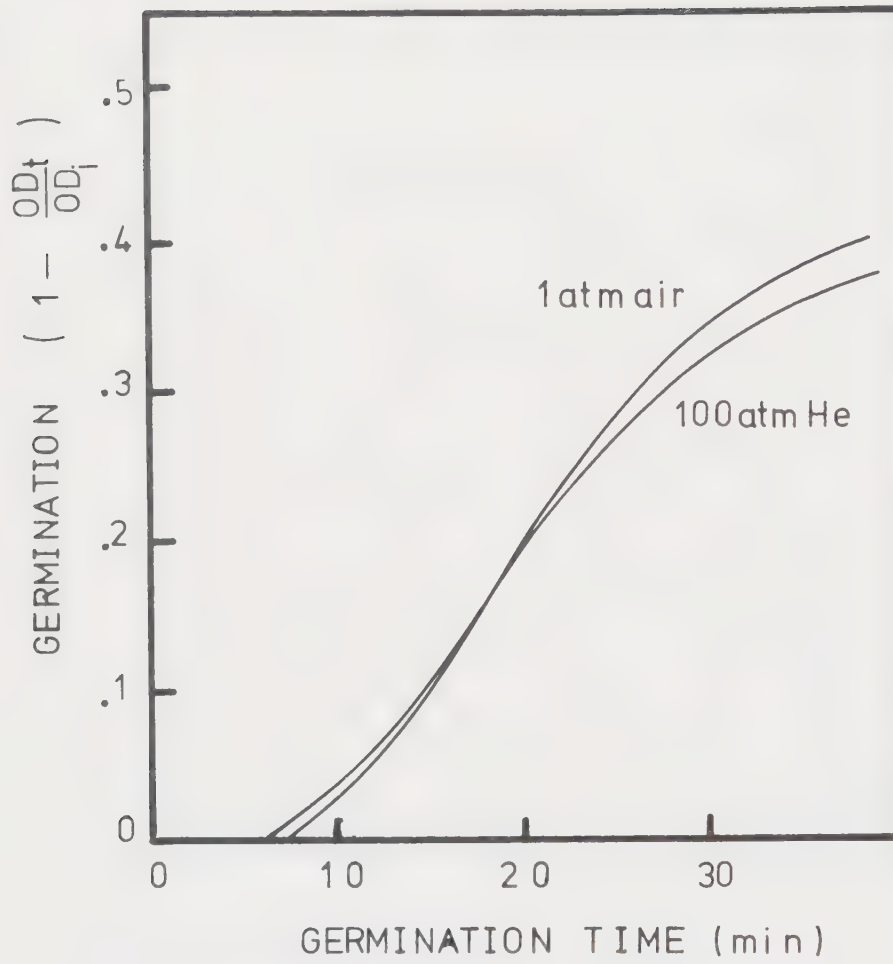


FIG 5. GERMINATION OF NON-ACTIVATED SPORES OF B. CEREUS AFTER EXPOSURE TO 1 ATM OF AIR AND 100 ATM OF He DURING 24 HOURS.

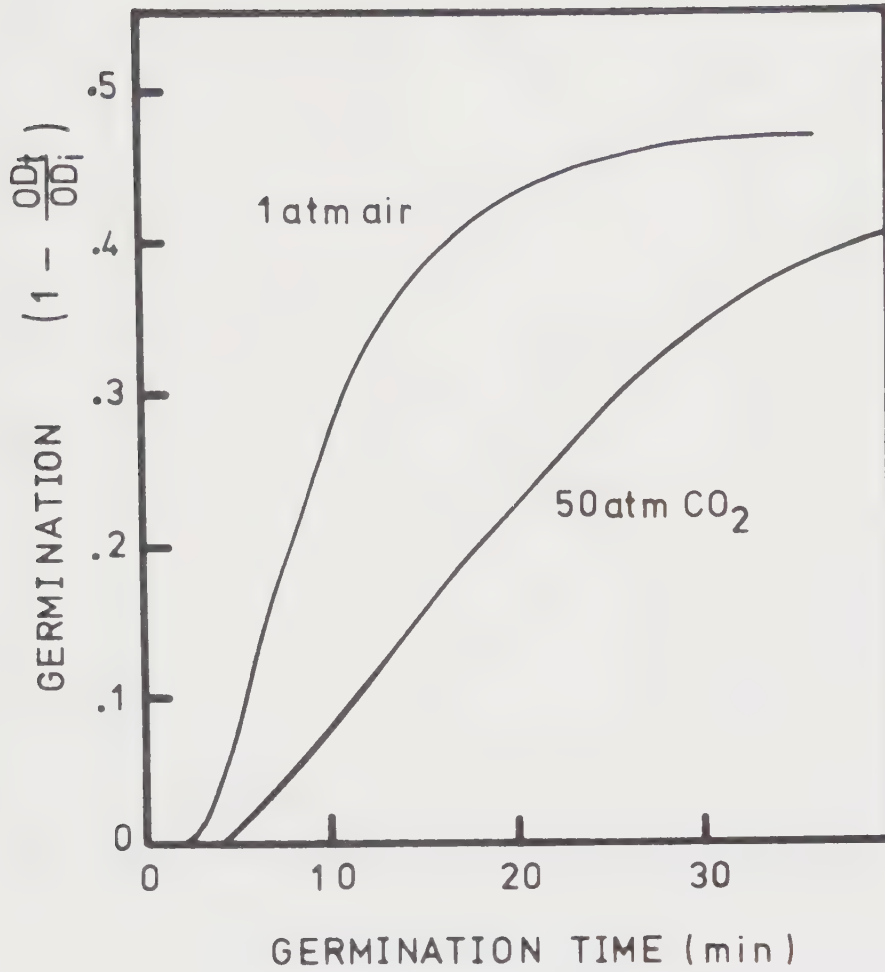


FIG 6. GERMINATION OF *B. CEREUS* SPORES AFTER INCUBATION IN 1 ATM OF AIR AND 50 ATM OF CARBON DIOXIDE DURING 24 HOURS AND SUBSEQUENT HEAT ACTIVATION.

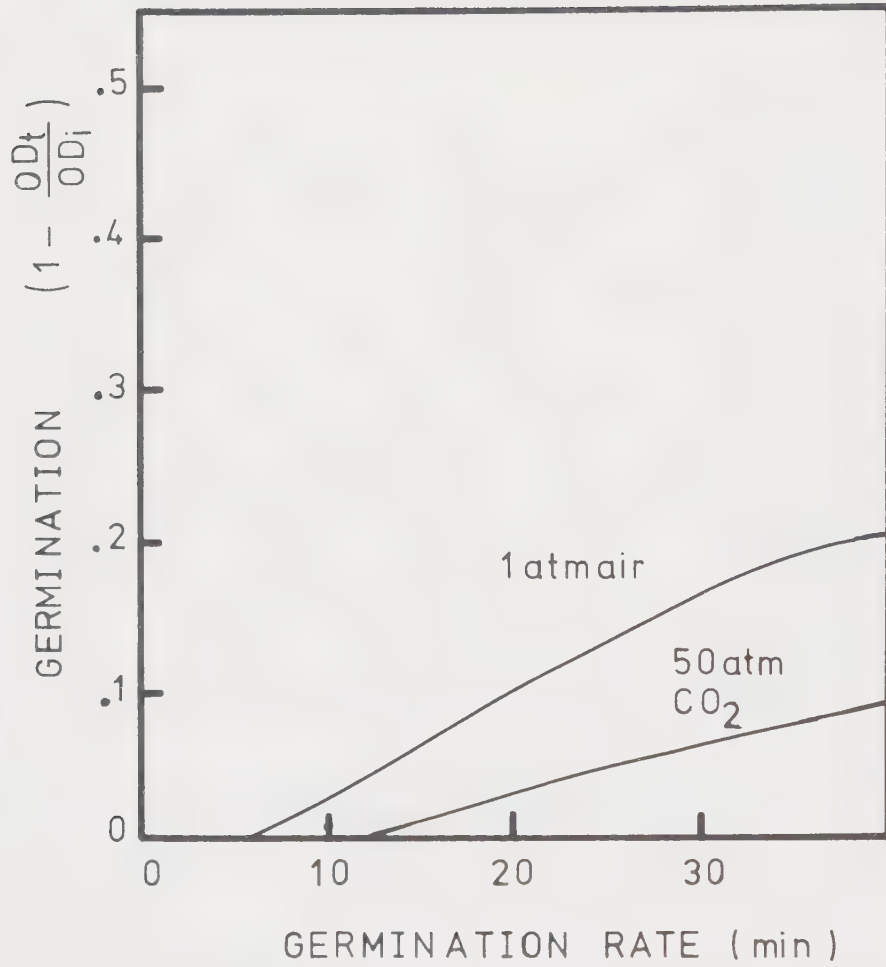


FIG 7. GERMINATION OF NON-ACTIVATED SPORES OF *B. cereus* AFTER EXPOSURE TO 1 ATM OF AIR AND 50 ATM OF CARBON DIOXIDE DURING 24 HOURS

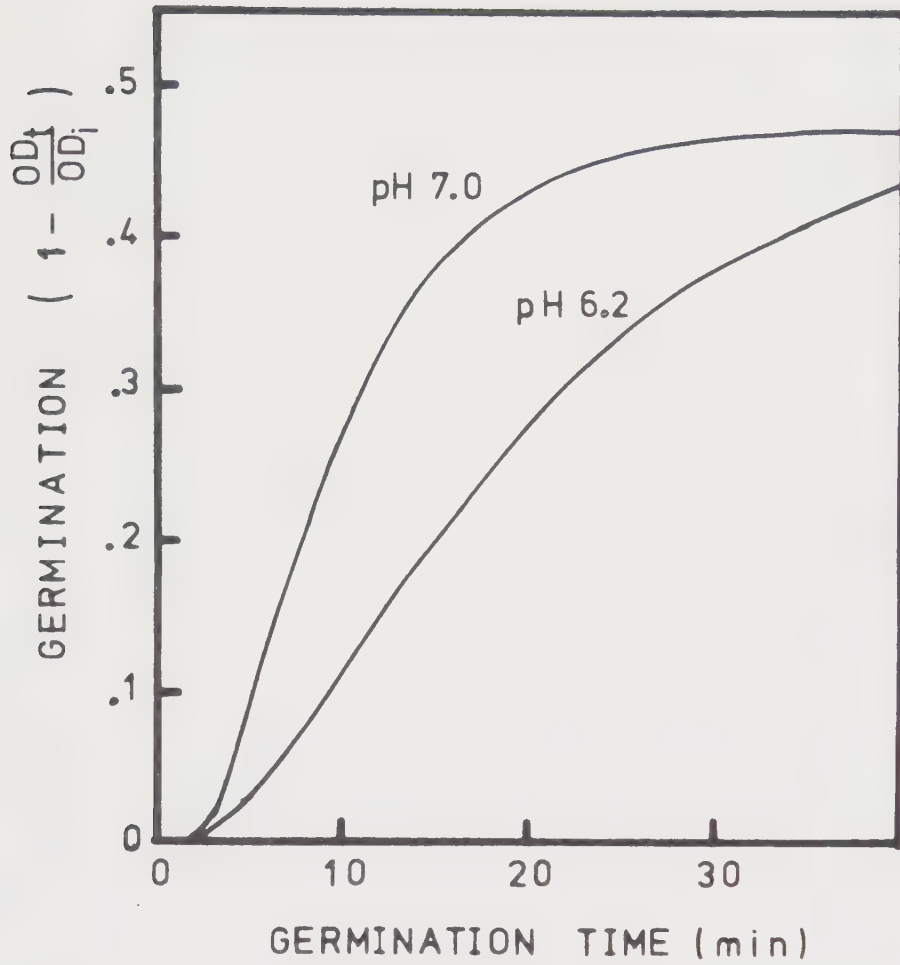


FIG 8. GERMINATION OF *B. CEREUS* SPORES IN 0.067 M PHOSPHATE BUFFER AT PH 7.0 AND 6.2.

INHIBITION OF THE GERMINATION OF BACILLUS CEREUS SPORES BY HIGH GAS PRESSURE

Summary

The effects of the gases helium, oxygen, nitrogen, air, argon, nitrous oxide, and carbon dioxide on the germination of B. cereus spores were investigated. Washed spore suspensions in phosphate buffer were equilibrated with a gas phase under pressure (max 100 atm) whereafter the germination was initiated by the addition of L-alanine and adenosine. Samples were intermittently taken from the pressure vessel and the germination was measured according to the spectrophotometric method. The germination under high pressure of the gases was inhibited in a way that suggests that it is the physical rather than the chemical properties of the gas that determine the inhibitory potential. Helium at 1 - 100 atm had no detectable effect on the germination and the inhibitory potential of the other gases could be arranged in the order $\text{He} < \text{air} = \text{N}_2 < \text{Ar} < \text{N}_2\text{O} < \text{CO}_2$. The inhibitory effect increased with increasing gas pressure. The inhibition was partly reversible and the mechanism of the inhibition was not merely an effect on the germination medium or on the spores. Inhibition of spore germination by bicarbonate is discussed in context with the carbon dioxide inhibition.

Introduction.

It has been shown previously in this thesis that 50 atm of air inhibits slightly the germination of Bacillus cereus spores but that the exposure to 100 atm of air during 1 day does not cause any changes in the subsequent kinetics of the germination. That means that the spore itself is very resistant to high air pressure but that the process of germination is somewhat sensitive. One might draw the conclusion that this inhibition is caused by the high oxygen partial pressure or by the hydrostatic pressure. However, hydrostatic pressure has been shown to initiate spore germination rather than inhibit it (64, 65, 66, 67) and no control was made in the experiment with 50 atm of air to exclude any effects of nitrogen.

Chemically inert gases like noble gases and nitrogen may exert effects on biological systems. These effects are caused by their physical rather than by their chemical properties. The most wellknown phenomenon is the so called inert gas narcosis. However, it has been shown that these gases have a more or less pronounced depressive effect on higher organisms as well as on microorganisms (144, 150, 145, 153). The potency to inhibit life reactions and to cause narcosis increases with the molecular weight and with the partial pressure of the gas. Thus, the heavier the gas, the lower is the partial pressure that is required to demonstrate the effect.

It is difficult to give any definite figures for the anesthetic or inhibiting pressures of the gases since these vary with the object and with the method of investigation. As a general rule, however, it could be mentioned that in the lightest gases, hydrogen and helium, no narcosis has been observed at pressures below 100 atm. At higher pressures the effects of the hydrostatic pressure often becomes critical. When neon, nitrogen or argon is used, pressures above 1 atm are required to cause narcosis. As for the heavy gases nitrous oxide, krypton and xenon pressures below atmospheric are anesthetic.

Oxygen is seldom mentioned in these investigations since it becomes strongly toxic at pressures much below those that could be expected to cause these phenomena (98). The same circumstances appear as for carbon dioxide since it is difficult to recognise its chemical effects from its physical ones.

There are several theories which explain the inert gas effects on cells from different standpoints and they do not necessarily exclude each other. Many investigators consider an interference with membranes resulting in changed electrolyte permeability, to be the reason for these effects (159, 142). In two similar theories (143, 160), the formation of gas hydrates is considered to be of great importance in the generation of narcosis. This idea is supported by the close correlation between anesthetic potency of the gas and the dissociation pressure of the gas hydrate. Spatial competition with oxygen has also been suggested as a mechanism for the inert gas effects (151).

This investigation was undertaken to see if the previous finding, that high air pressure inhibits the germination of Bacillus cereus spores, was caused by the high oxygen partial pressure, by the high nitrogen pressure or by the hydrostatic pressure. Since the experiments very soon showed that the nitrogen-atmosphere, used as a control for oxygen effects, was as inhibitory as air at the same pressure, the investigation was extended to cover some other gases.

Material and methods

Spores. Spores of Bacillus cereus (SIK strain No 216) were prepared from shake flask culture prior to each experiment as described in the previous chapter. When free from sporeangia the spore suspension was washed three times with 67 mM phosphate buffer (pH 7.0) and resuspended in the buffer and kept in a refrigerator until the time of the experiment (within one week). Just before the experiment the spores were washed once more in the buffer and resuspended to $OD^{620} \sim 0.8$. This suspension was heat activated for 15 min. at $80^{\circ}C$ and used for the germination experiments.

Gases. Carbon dioxide was purchased from AB Kolsyrefabrikerna, Lidingö, and the other gases were purchased from AB Aga, Lidingö. The oxygen impurities of some of the gases were analysed with a gas chromatograph (Molecular sieve 5A and thermistor detector). The following oxygen contents in per cent (v/v) were obtained: in He $< 10^{-3}$, in N_2 0.02, in N_2O 0.45 and in CO_2 0.06. The oxygen content of argon could not be analysed with this method but should be less than $2 \cdot 10^{-3}\%$ according to the producer.

Germination initiator. As germination initiator 2 mM adenosine and 10 mM L-alanin was used.

Pressure treatment. 200 ml of the spore suspension was transferred to a thermostated ($30^{\circ}C$) pressure vessel (500 ml volume) furnished with a mixer, a sparger, and valves for gas inlet, gas outlet, and sampling. (fig. 1 on page 76). The gas was flushed through the vessel for a quarter of an hour in order to remove the air and then the gas pressure was applied for an hour to allow equilibration between the dissolved gas and the gas phase.

The germination was started by crushing a glass ampoule with the germination initiator, which was placed inside the pressure vessel.

Control. As control a sample was taken from the pressure vessel just before the onset of the germination. It was vigorously aerated for 30 seconds and the germination initiator was added. The OD^{620} was then recorded continuously in a thermostated ($30^{\circ}C$) cuvette of a spectrophotometer.

Germination measurement. After the onset of the germination samples were taken intermittently from the pressure vessel and the OD^{620} was measured. These samples were aerated vigorously and the subsequent germination in normal atmosphere was followed by intermittent or continuous measurement of the OD^{620} . The germination was occasionally verified by microscopic examination in phase contrast microscope.

Calculations. The germination has been expressed in terms of the function

$1 - \frac{OD_t}{OD_i}$, where OD_t is the optical density at 620 nm at time "t" and OD_i is the initial optical density. The relative number of germinating spores has been expressed as $1 - \frac{OD_f}{OD_i}$, where OD_f is the final optical density mostly reached within 30 - 60 min. The rate of germination has been expressed

as the derivative $\frac{d(1 - \frac{OD_t}{OD_i})}{dt}$, at the inflexion point of the curve. The germination rate was also measured as t_{50} , which means the time which was needed to reach 50% of $1 - \frac{OD_f}{OD_i}$. Running parallel with each experiment a control germination in air has been performed. Since these control curves have not been identical throughout the investigation all germination data have been expressed as index in which the value of the sample is given in per cent of the corresponding value of the control.

Results

Most experiments have been repeated one or several times. A summary of the results is shown in table I. The index figures are mean values. In order to illustrate its course the germination curve from one typical experiment in each atmosphere has been selected and shown in figure 1 and 2. The points

indicate observations. In curves without any points the measurement has been continuous.

As shown in figure 1 and table I helium does not influence the germination appreciably at 1 or 100 atm. Air and nitrogen at 100 atm inhibit the germination with respect to the number of germinating spores as well as to the rate of germination. Experiments were also performed at 21 atm of pure oxygen in order to check whether the high oxygen partial pressure of 100 atm of air might influence the germination. This oxygen pressure is much higher than what is needed to severely injure most living organisms but it did not interfere appreciably with any of the measured germination parameters.

Argon did not influence the germination at 1 atm. However, at 5 atm an inhibition of the rate of germination could be detected, and at 50 atm no germination occurred at all (figures 2 and 3).

The only gases that inhibited the germination at atmospheric pressure were nitrous oxide and carbon dioxide. 1 atm of carbon dioxide inhibited the germination completely during 24 hours in 4 experiments. In two experiments, however, about 20% of the spores germinated at a very slow rate.

A series of experiments was made with argon and carbon dioxide to elucidate the effect of increased partial pressure of the inhibiting agent. The para-

meters $1 - \frac{OD_f}{OD_i}$ and $\frac{d(1 - \frac{OD_t}{OD_i})}{dt}$ from these experiments are shown in figures 3 and 4 and table II. The carbon dioxide experiments were performed at 1 atm gas pressure with the carbon dioxide diluted with air or helium to the desired partial pressure.

In order to check effects of pH changes during the carbon dioxide experiments a series of tests were performed. Firstly the influence of carbon dioxide pressure on the buffer was measured. The results are given in fig. 5. Apparently the pH was depressed by the gas treatment. Secondly the effect of pH on the germination was measured (see fig. 6). It is evident that the rate of germination but not the extent of germination was influenced by the pH (fig. 7). The parameters in fig. 4 are corrected with respect to the pH effect on germination of the control.

The effect of sodium bicarbonate on the germination was also studied in two experiments. In each experiment one control and two samples with respectively 3 and 100 mmol NaHCO_3 per litre buffer was run. The inhibitory effect was slight in 3 mM HCO_3^- and somewhat more evident in 100 mM HCO_3^- (fig. 8).

Investigations were also made to study the nature of the inhibition. Samples were taken from spore suspensions that were totally or partly inhibited, and the subsequent germination in 1 atm of air was measured. An illustration of these subsequent germinations is shown in figure 9. In the illustrated experiment a spore suspension that was partly inhibited by 25 atm of air was used. 50 min. after the addition of the germination initiator, about 40% of the spores had germinated and no further germination occurred in the pressure vessel during a period of 18 hours. Samples were taken from the pressure vessel after 50, 75, and 130 min. and after 18 hours. These suspensions were further incubated in normal air atmosphere, and then samples taken during the first 50 min. after the onset of the germination germinated at a normal rate to the same extent as did the control. However, samples taken at 75 min. or later showed an increasing reduction of the extent as well as of the rate of the germination. No additional germination occurred at all when, after 18 hours in the pressure vessel, the sample was transferred into normal air atmosphere. If, however, another dose of germination initiator was added to these samples (at the arrows in figure 9) the germination was completed. Similar results were obtained in most, but not all, experiments with partial or complete inhibition of the germination.

In order to investigate whether the inhibition was caused by an effect of the gas on the germination initiator, a solution of this was incubated during 24 hours in 1 atm of carbon dioxide and in 50 atm of argon. Each of these solutions was then used in germination experiments in normal air atmosphere. No difference was detected when untreated germination initiator was replaced with the one treated with gas.

Discussion

It is evident from the results (table I) that the germination process in B. cereus spores is sensitive to high pressure of all the gases except helium.

Furthermore, the sensitivity pattern corresponds to that of the general inhibiting and anesthetic effects of these gases. The hydrostatic pressure does not seem to influence the germination at 100 atm as can be concluded from the germination in 1 and 100 atm of helium. Nor did the helium itself have any inhibitory effect on the germination at 100 atm. This is in accordance with the general theory that states that the lightest gases do not even show any inhibitory effects on life processes at pressures of about 100 atm. Actually, as complex beings as mice can live at 125 atm of helium without any signs of narcosis (88).

It was tempting to believe that the high oxygen partial pressure (21 atm) was responsible for the inhibition in the 100 atm air experiment. The control experiment with pure oxygen at 21 atm showed clearly that this was not so. This is further proved by the fact that 100 atm of nitrogen exerted almost exactly the same effect as 100 atm of air (table I).

Thus, it can be stated that B. cereus spores as well as their germination are extremely resistant to oxygen at high pressure compared with vegetative cells.

Nitrous oxide and carbon dioxide were the only gases tested that caused inhibition at atmospheric pressure. In the case of 1 atm of carbon dioxide there was some germination in two of six experiments. This is probably due to the fact that 1 atm of carbon dioxide seems to be the limit of tolerance for the germination (figure 4). In the test nitrous oxide had an inhibitory capacity between argon and carbon dioxide but its molecular weight is the same as that of carbon dioxide. Of course it is not the molecular weight itself that provokes the inhibitory potency of the gas but rather some physical properties that vary with the molecular weight. In the case of nitrous oxide and carbon dioxide it is possible that even chemical effects have an influence on the germination.

The difficulties in excluding effects of chemical origin in the carbon dioxide and nitrous oxide experiments made a test with argon which is a true inert gas very interesting. In figure 3 it is clearly shown that increased argon pressure has an inhibitory effect on the germination. It decreased both the rate of germination and the number of germinating spores.

It is evident from Fig. 3 and still more from Fig. 4 that the rate of germination is the most sensitive parameter. Table I and II also show that the parameters t_{50} and lag time were not very discriminating between the different gas atmospheres.

It can be concluded from the experiments that the inhibition of the germination of B. cereus spores is not just a result of oxygen deficiency. Indeed, there were impurities of oxygen but if the inhibitions were results from oxygen deficiency then an increased gas pressure should have resulted in less inhibition. Further evidence is found in the results from the carbon dioxide experiments shown in figure 4. If oxygen deficiency was the reason for the inhibition then no inhibition should have been found in the experiments with 0.5 - 0.9 atm of carbon dioxide. Another evidence are the results of the germination in 0.5 atm helium and 0.5 atm carbon dioxide. In this atmosphere the germination was approximately the same as that in 0.5 atm air and 0.5 atm carbon dioxide.

The effect of sodium bicarbonate on the germination of some physiologically different Bacillus species has been described in literature (196). It was found that the germination of these spores from all species tested were slightly inhibited by 5 - 50 mM sodium bicarbonate. The inhibitory pattern of this treatment was similar but weaker than the effect by carbon dioxide found in this investigation. There is a good possibility that the origin of the inhibitory effects of carbon dioxide and bicarbonate is common.

Since the inhibitory effect of carbon dioxide fits the theory of the general biological inhibitory effect of "inert" gases on basis of their physical properties it might be concluded that the inhibitory effect of bicarbonate is caused by the carbon dioxide that will be generated at the dissolution of bicarbonate in water. In order to test this hypothesis the concentration of dissolved carbon dioxide in the bicarbonate experiments must be known. No figures are available since the experimental conditions have been different, but an approximative calculation can be done: suppose that the solubility of carbon dioxide in the experimental solutions is the same as in water at 25°C, 34 mM. (The actual value is lower.) Then dissolved carbon dioxide concentration can be calculated in all experiments. This has been done in table III. The concentration values are corrected with respect to

the actual pH. The degree of optical density reduction after 30 min in percentage of the corresponding value of the control, has been plotted against the calculated dissolved carbon dioxide concentrations for the carbon dioxide experiments as well as for the sodium bicarbonate experiments. The result gives some indication that the inhibitory effect of the bicarbonate might be caused by the carbon dioxide.

The nature of the inhibition was also studied in all cases where inhibition was found. It was reversible when the time of treatment with the inhibitory agent had not been too long. When this time was extended, the rate of germination and the number of germinating spores in most experiments decreased (figure 9). When the treatment with the inhibiting agent was extended overnight no additional germination occurred at all at the transferring of the spores to 1 atm of air. The fact that an additional dose of germination initiator could restart the germination so that it was accomplished in a normal way indicates that the inhibition interferes with the germination initiators. However, no inactivation of the initiators was found when these were subjected to 1 atm of carbon dioxide or 50 atm of argon, atmospheres that effectively inhibited the germination.

The spores might be another possible target for the gas. However, in the previous investigation it was shown that the spores, without germination initiator, could be exposed during long time to atmospheres that in the present investigation have been shown to be inhibitory.

TABLE I.

Atmosphere	Number of exp.	Extent of germination $1 - \frac{OD_f}{OD_i}$	Rate of germination		Lag time
			$\frac{d(1 - \frac{OD_t}{OD_i})}{dt}$	t_{50}	
1 atm He	4	96	93	120	100
100 atm He	2	107	110	100	< 200
1 atm N ₂	3	88	60	140	100
100 atm N ₂	5	41	17	260	250
50 atm air	2	70	100	60	< 200
100 atm air	4	41	34	200	250
21 atm O ₂	2	94	69	250	200
1 atm Ar	2	100	100	100	100
5 atm Ar	1	101	80	130	< 200
15 atm Ar	2	90	51	171	200
25 atm Ar	2	44	20	260	300
35 atm Ar	1	16	9	300	300
50 atm Ar	2	0	0	-	-
1 atm N ₂ O	2	64	15	350	300
1 atm CO ₂	6	0	0	-	-
50 atm CO ₂	3	0	0	-	-

EFFECTS OF SOME GAS ATMOSPHERES ON THE GERMINATION OF B. CEREUS SPORES. THE GERMINATION PARAMETERS ARE EXPRESSED IN PERCENTAGE OF THE CORRESPONDING VALUE OF THE CONTROL IN 1 ATM OF AIR.

TABLE II.

GAS COMPOSITION	NUMBER OF EXP.	EXTENT OF GERMINATION $1 - \frac{OD_f}{OD_i}$	RATE OF GERMINATION		LAG TIME
			$\frac{d(1 - \frac{OD_t}{OD_i})}{dt}$	t_{50}	
100% CO ₂	6	0	0	-	-
90% CO ₂ + 10% AIR	2	24	5.1	750	730
75% CO ₂ + 25% AIR	2	63	6.9	930	530
50% CO ₂ + 50% AIR	2	87	12.8	630	500
50% CO ₂ + 50% HE	1	91	17.6	630	470

EFFECTS OF CARBON DIOXIDE ON THE GERMINATION OF B. CEREUS SPORES. THE GERMINATION PARAMETERS ARE EXPRESSED IN PERCENTAGE OF THE CORRESPONDING VALUE OF THE CONTROL IN 1 ATM OF AIR. ALL EXPERIMENTS WERE PERFORMED AT 1 ATM GAS PRESSURE.

TABLE III.

INHIBITING AGENT	pH	TOTAL CONC OF H_2CO_3 (mM)	DISSOLVED CO_2 (mM)	EXTENT OF GERMINATION AT 30 MIN (% OF CONTROL)
0.5 ATM CO_2	6.40	17	6.3	54; 60
0.75 ATM CO_2	6.30	26	11.2	21; 25
0.90 ATM CO_2	6.25	31	14.1	10; 11
1.0 ATM CO_2	6.20	34	17.8	0
3 mM HCO_3^-	7.20	3	0.5	89
100 mM HCO_3^-	7.90	100	2.0	80

COMPARISON BETWEEN THE INHIBITION OF GERMINATION OF B. CEREUS SPORES AND A CALCULATED CONCENTRATION OF DISSOLVED CARBON DIOXIDE IN EXPERIMENTS WITH CARBON DIOXIDE AND SODIUMBICARBONATE. THE RELATION IS PLOTTED IN FIG 10.

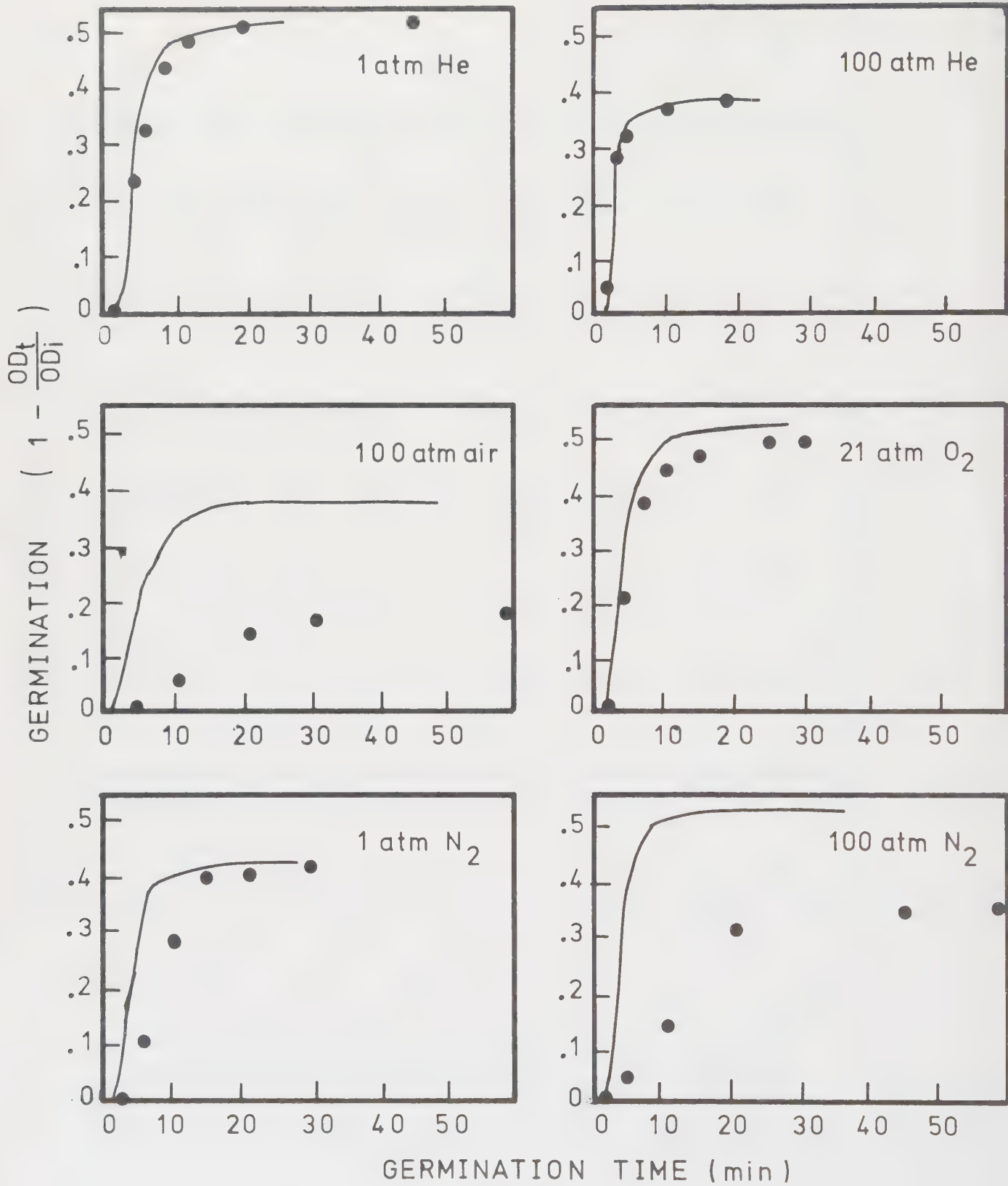


FIG 1. EFFECTS OF SOME GAS ATMOSPHERES ON THE GERMINATION OF *B. cereus* SPORES. THE CONTINUOUS LINES INDICATE THE CONTROL IN 1 ATM OF AIR. THE POINTS INDICATE GERMINATION IN THE PRESSURE VESSEL.

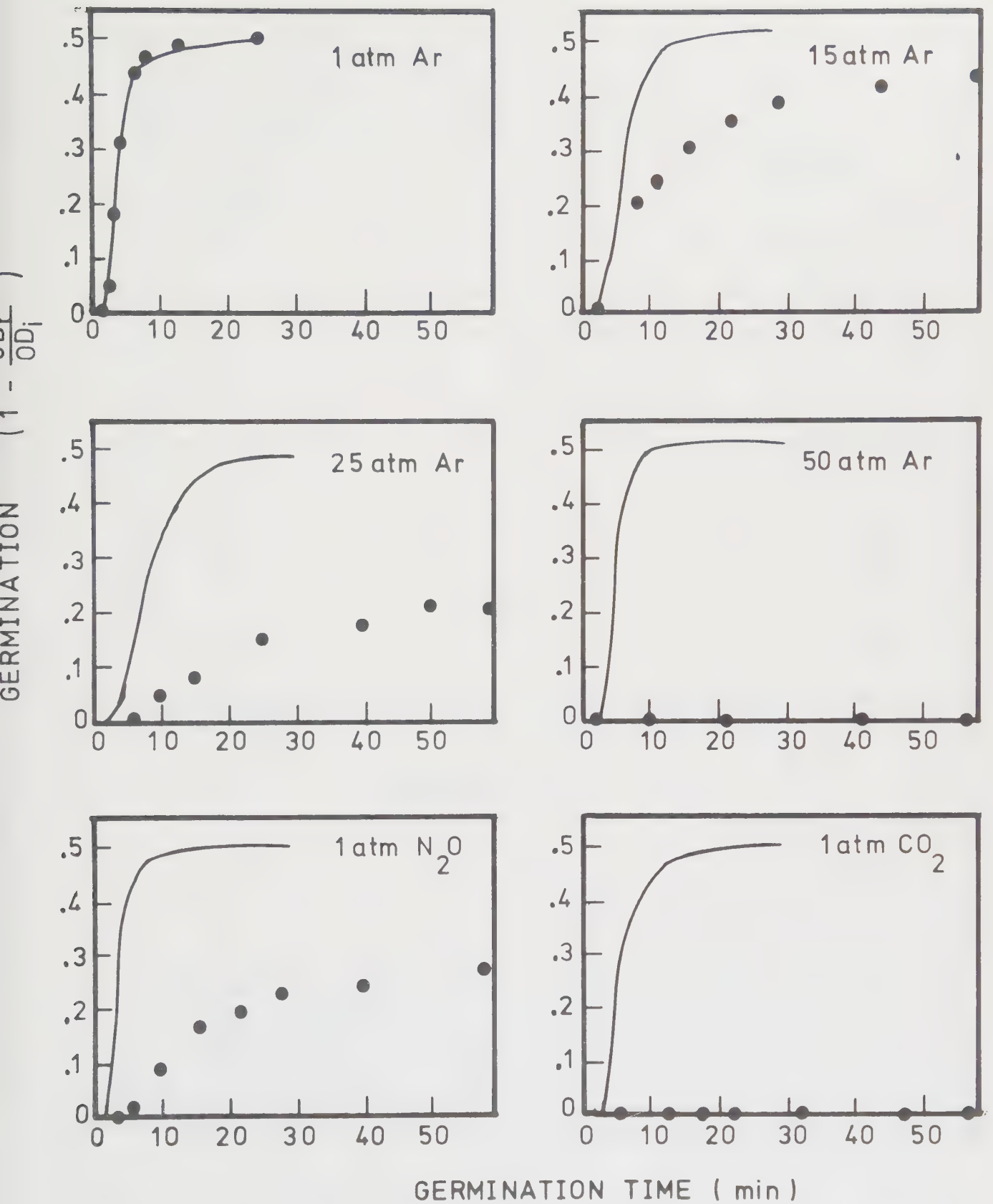


FIG 2. EFFECTS OF SOME GAS ATMOSPHERES ON THE GERMINATION OF *B. cereus* SPORES. THE CONTINUOUS LINES INDICATE THE CONTROL IN 1 ATM OF AIR. THE POINTS INDICATE GERMINATION IN THE PRESSURE VESSEL.

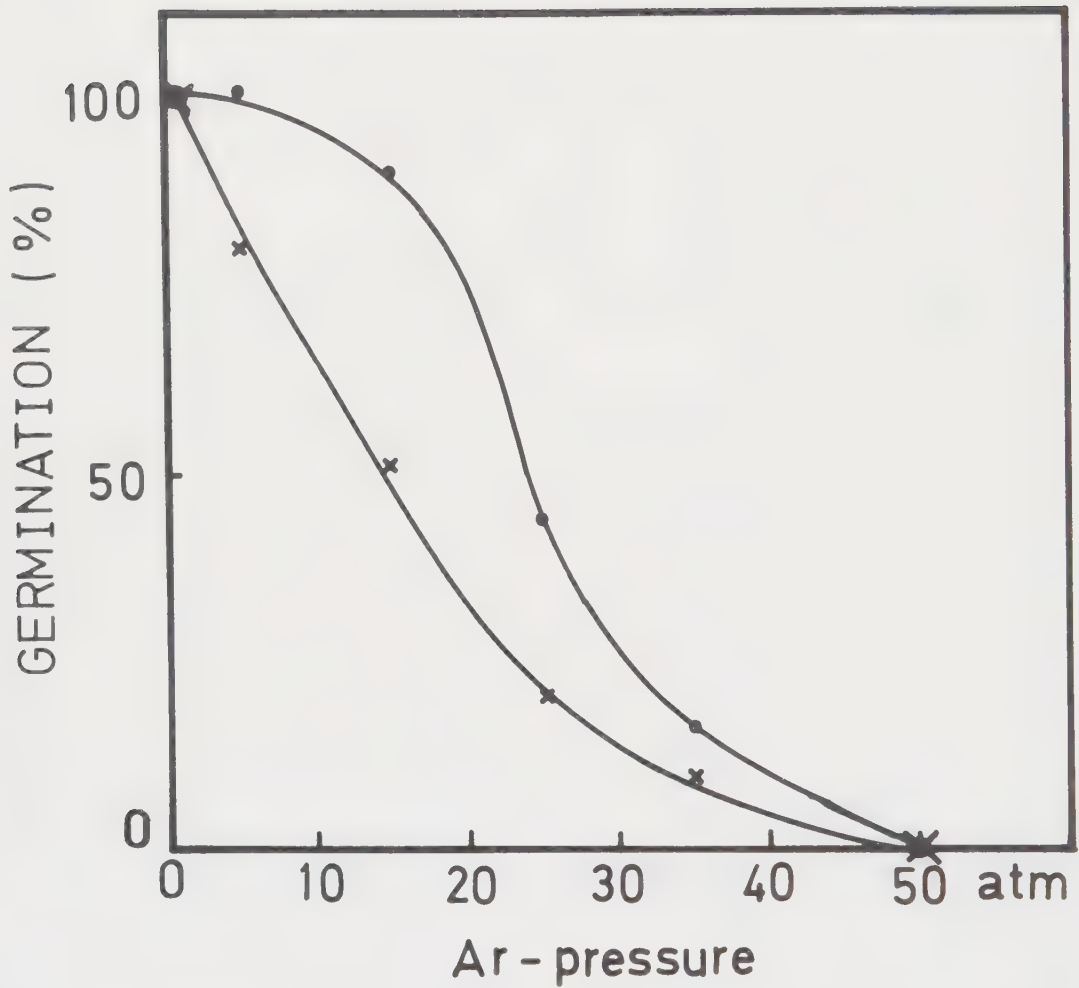


FIG 3. THE EFFECT OF ARGON PRESSURE ON THE GERMINATION OF *B. CEREUS* SPORES. THE EXTENT OF GERMINATION IN TERMS OF $1 - \frac{OD_f}{OD_i}$ (●—●) AND THE RATE OF GERMINATION IN TERMS OF $d(1 - OD_t/OD_i)/dt$ (x—x) ARE GIVEN IN PERCENTAGE OF CORRESPONDING VALUE OF THE CONTROL.

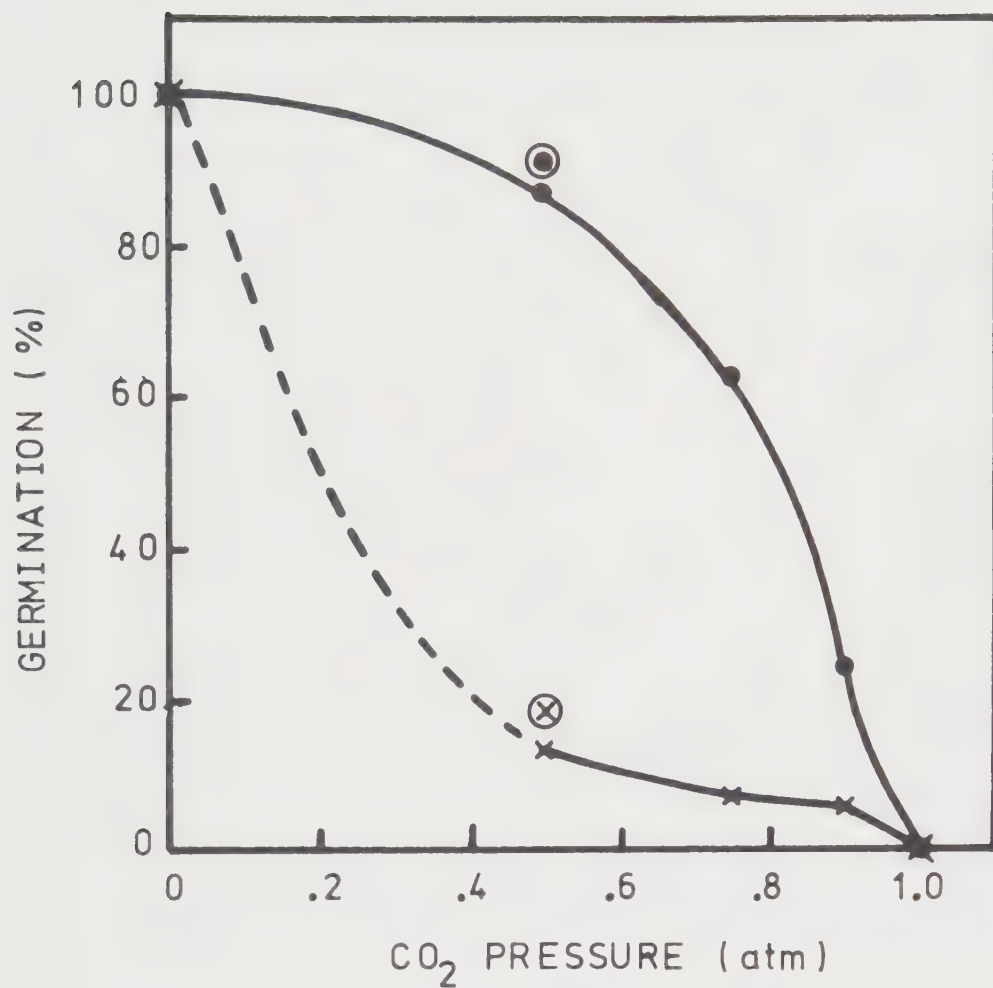


FIG 4. THE EFFECT OF CARBON DIOXIDE PRESSURE ON THE GERMINATION OF *B. CEREUS* SPORES. THE EXTENT OF GERMINATION IN TERMS OF $1 - OD_f / OD_i$ (●—●) AND THE RATE OF GERMINATION IN TERMS $d(1 - OD_f / OD_i) / dt$ (×—×) ARE GIVEN IN PERCENTAGE OF CORRESPONDING VALUE OF THE CONTROL.

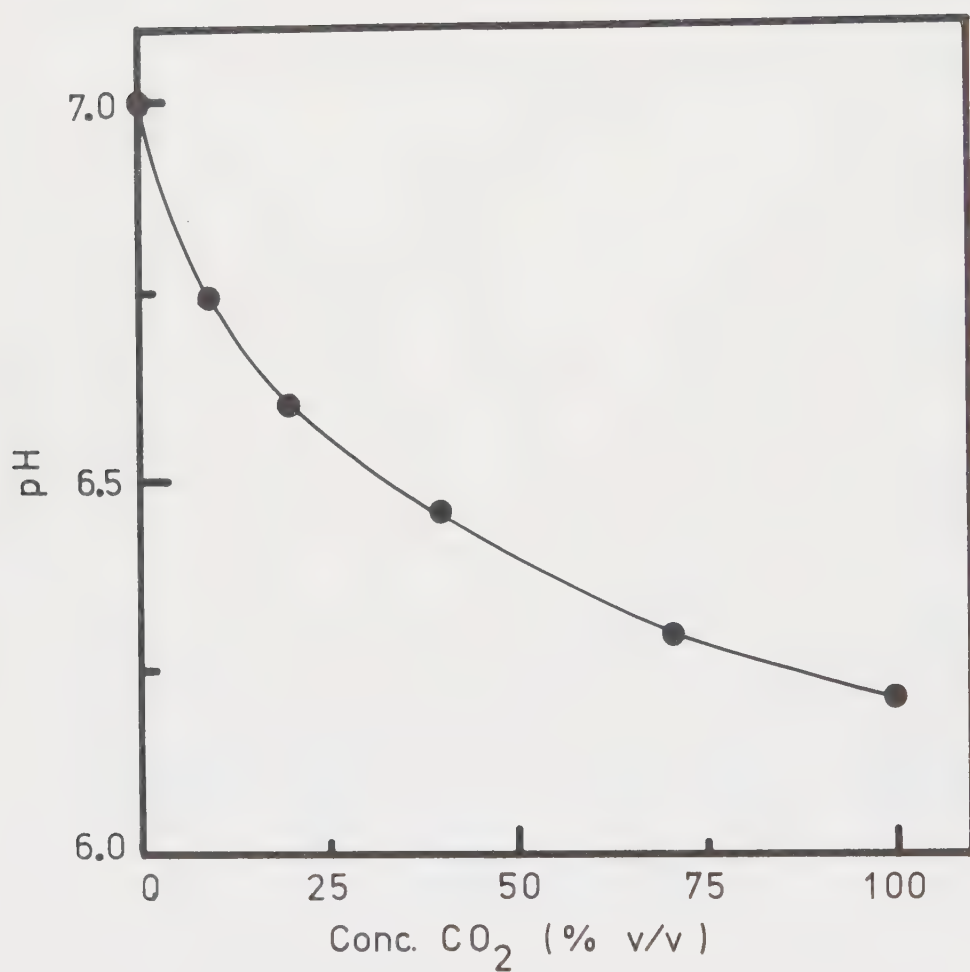


FIG 5. THE EFFECT OF THE CARBON DIOXIDE CONCENTRATION IN AIR ON THE pH OF 0.067 M PHOSPHATE BUFFER WITH ORIGINAL pH 7.0. THE BUFFER WAS FLUSHED WITH AN AIR-CARBON DIOXIDE MIXTURE AND pH WAS MEASURED.

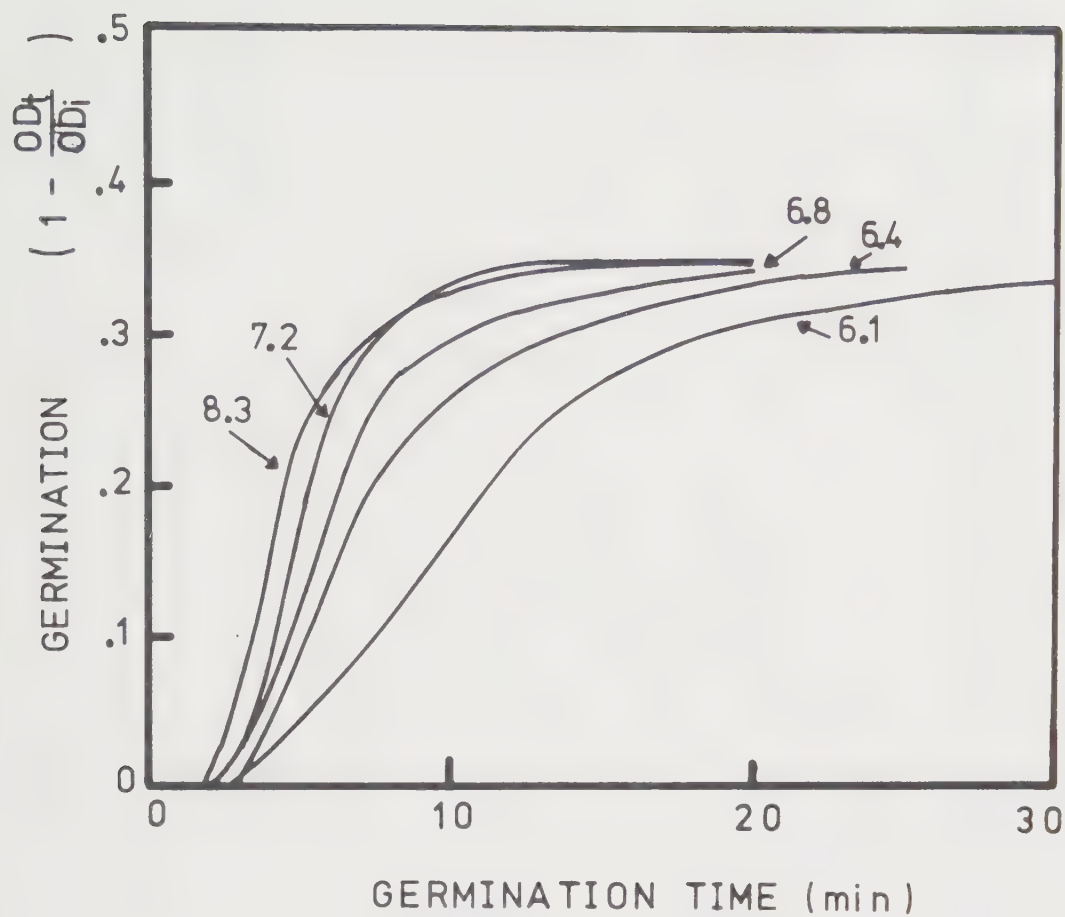


FIG 6. THE EFFECT OF pH ON THE GERMINATION OF *B. cereus* SPORES. THE COMPONENTS OF THE PHOSPHATE BUFFER WERE ADJUSTED TO GIVE DIFFERENT pH OF THE SOLUTIONS. THESE DATA ARE FURTHER INTERPRETED IN FIG 7.

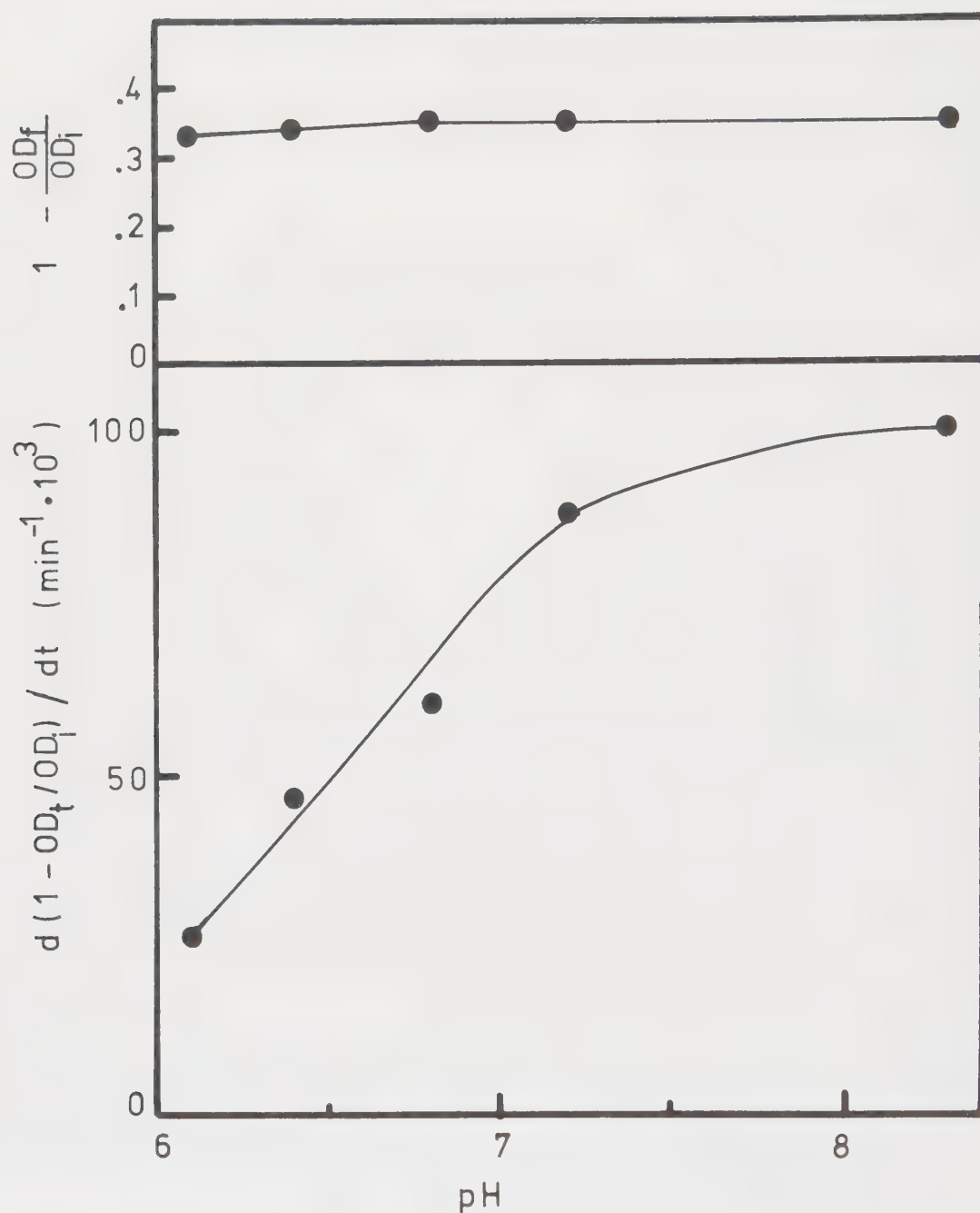


FIG 7. THE EFFECT OF pH ON THE RATE OF GERMINATION, MEASURED AS MAXIMUM RATE OF DECREASE IN OPTICAL DENSITY (LOWER CURVE), AND ON THE EXTENT OF GERMINATION, MEASURED AS THE FINAL DECREASE IN OPTICAL DENSITY (UPPER CURVE).

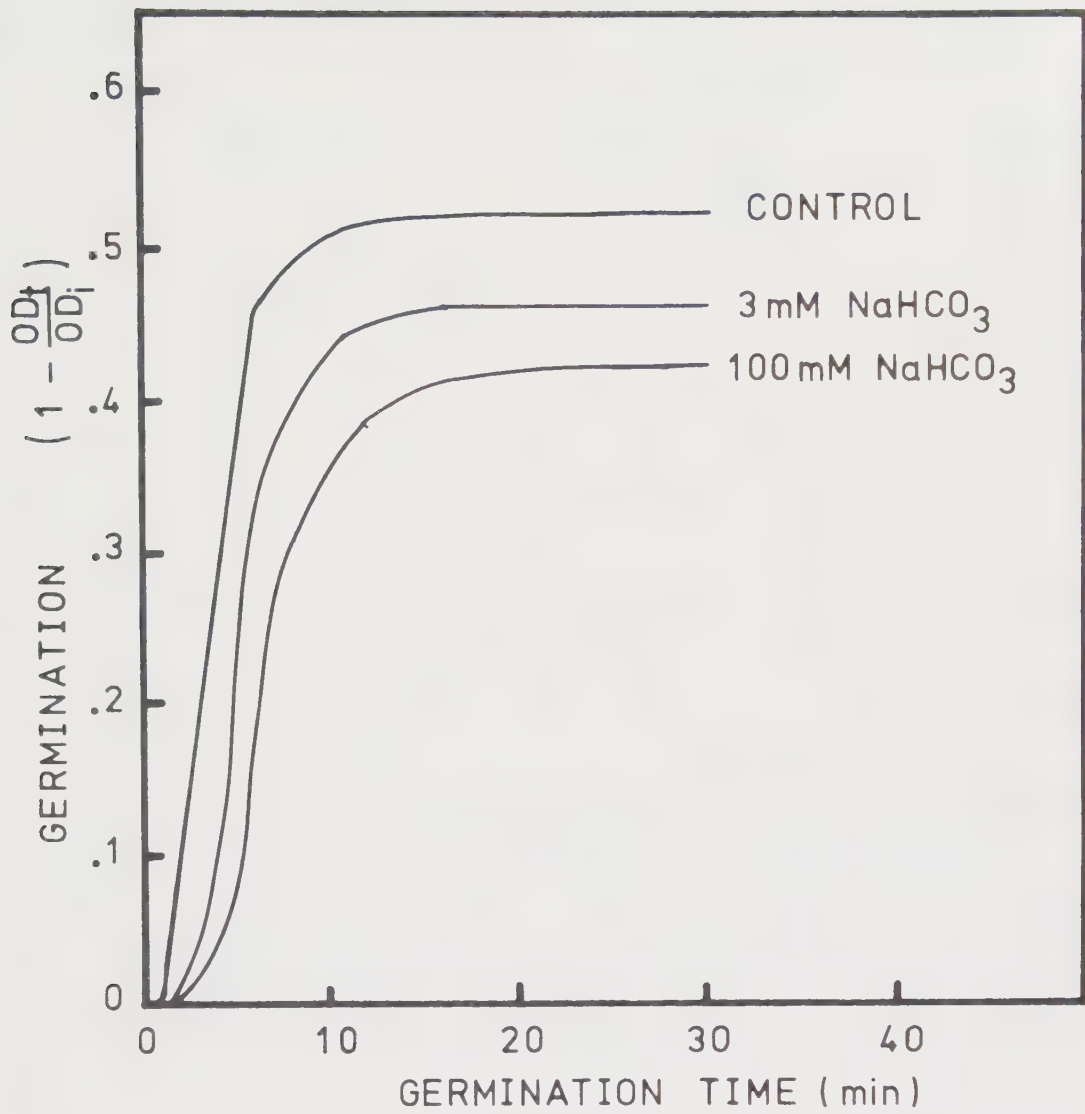


FIG 8. EFFECT OF SODIUM BICARBONATE ON THE GERMINATION OF B. CEREUS SPORES. THESE DATA ARE FURTHER INTERPRETED IN FIG 10 AND TABLE III.

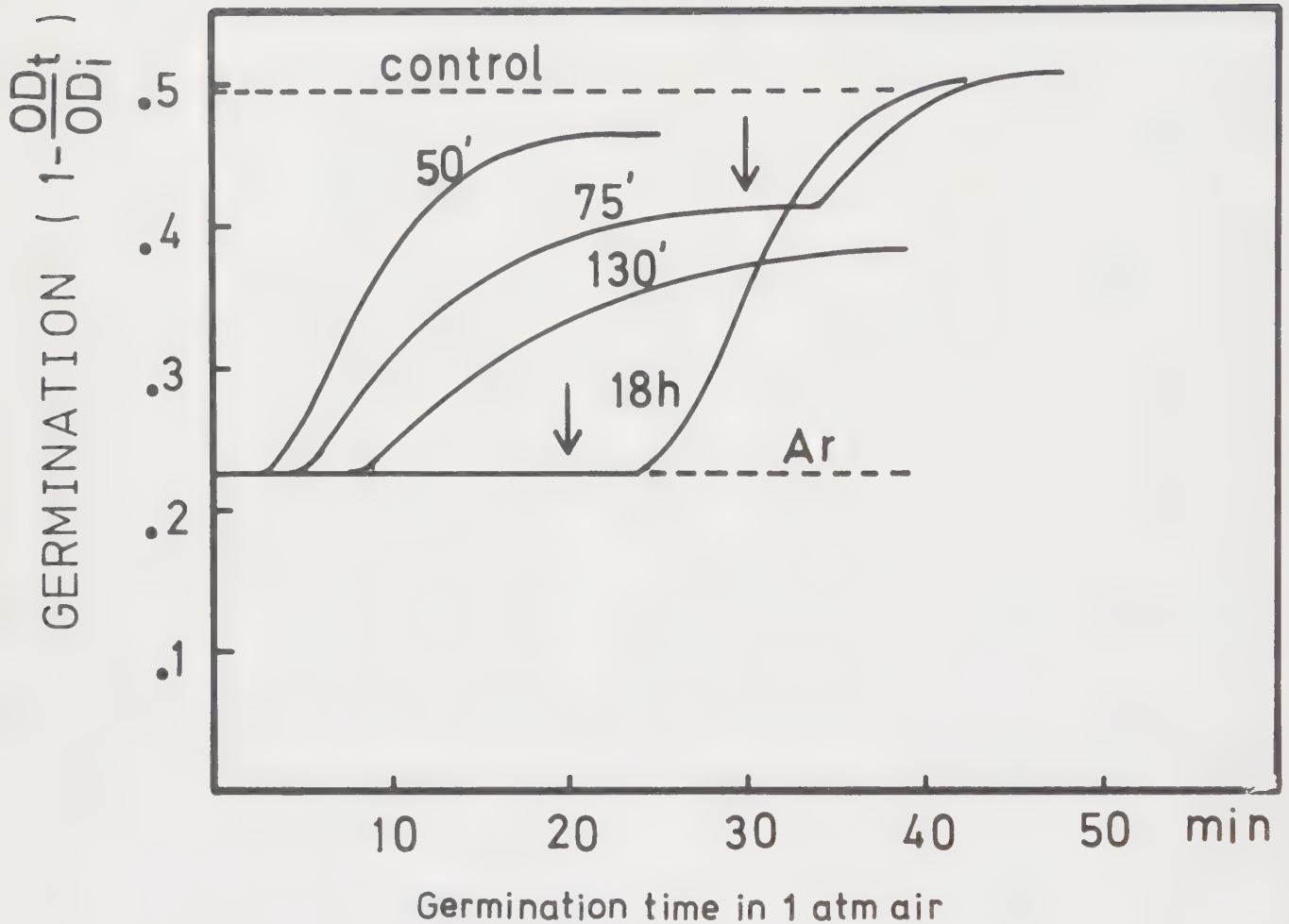


FIG 9. GERMINATION OF B. CEREUS SPORES THAT HAVE BEEN PARTLY INHIBITED BY 25 ATM OF ARGON AND THEN TRANSFERRED INTO NORMAL AIR ATMOSPHERE. THE FIGURES ON THE CURVES INDICATE AT WHICH TIME AFTER THE START OF THE GERMINATION IN 25 ATM OF ARGON THE SAMPLE WAS TRANSFERRED INTO AIR. AT THE ARROWS MORE GERMINATION INITIATOR WAS ADDED. THE FINAL LEVELS OF THE GERMINATION CURVES OF THE CONTROL AND OF THE GERMINATION IN 25 ATM OF ARGON ARE INDICATED BY DOTTED LINES.

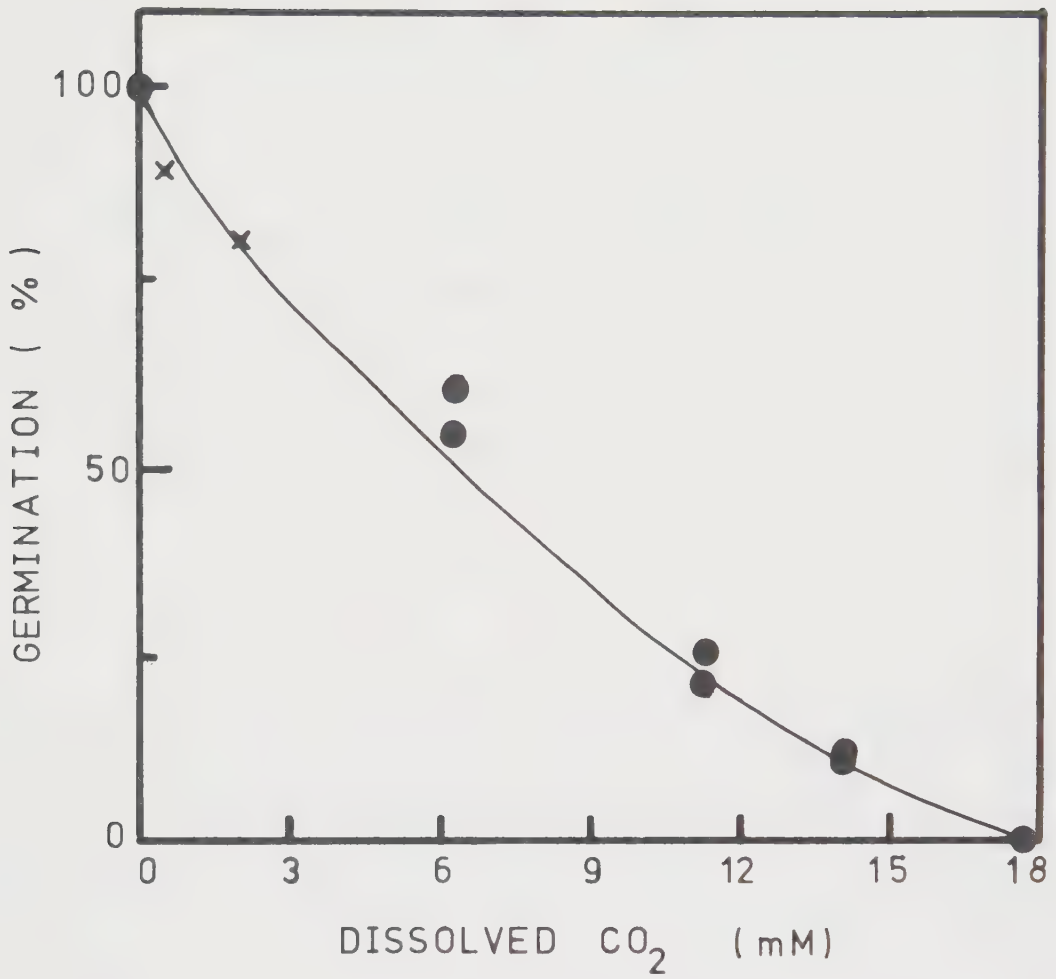


FIG 10. EFFECT OF THE CONCENTRATION OF DISSOLVED CARBON DIOXIDE (ESTIMATED AS DESCRIBED IN TEXT) ON THE EXTENT OF GERMINATION IN PERCENTAGE OF THE CONTROL. POINTS INDICATE EXPERIMENTS WITH GASEOUS CARBON DIOXIDE AND CROSSES INDICATE EXPERIMENTS WITH SODIUMBICARBONATE.

EFFECTS OF NOBLE GASES ON SPORE GERMINATION

Summary

The effects of xenon and krypton at pressures from 0.5 atm to 10 atm, on the germination of Bacillus cereus spores were investigated. Xenon at 1 atm inhibited slightly the germination. At more than 3 atm of xenon the inhibition was complete. Krypton exerted a similar but not as strong an effect. All inhibitions were reversible so that the germination recovered as soon as the inhibiting gas atmosphere was removed. The presence of air did not influence the inhibition. The nature of the inhibition of spore germination by noble gases and by some other gases like nitrogen, nitrous oxide and carbon dioxide is discussed in context with other known effects of these gases on biological reactions.

Introduction

It was shown in the previous chapter that argon, in spite of its chemical inertness, exerted inhibition of the germination of Bacillus cereus spores. The inhibiting potency increased with increasing pressure of argon and it was necessary to apply at least 5 atm to be able to notice the inhibition which was complete at 50 atm. No corresponding effect of helium at pressures up to 100 atm was found.

The finding that a noble gas can directly influence a life reaction is not unique. The so called inert gas narcosis has long been known (155) though its mechanism is still not completely understood. All noble gases, except helium, and some other gases like nitrogen, nitrous oxide, and methane have been shown to cause narcosis in man or animals. The heavier the gas the lower partial pressure of the gas is needed. Nitrogen becomes anaesthetic at 7 - 10 atm and argon at 3 atm. Xenon and nitrous oxide are anaesthetic at pressures below atmospheric (many data are collected in ref. 143 and 160).

Some theories are commonly referred to in the discussion on inert gas narcosis. However, there is nothing in these relations that limits the effects to just narcosis or effects on larger animals since the suggested mechanisms seem to function on a basal cellular level.

Enrichment of the gases in the lipids of membranes have been suggested as a mechanism of inert gas narcosis (154). This is supported by the close correlation between the partition coefficients for lipid/water and the anaesthetic potency of the gas. When the gases are dissolved in the lipid phase they may also interfere with the interface since they are known to increase the film pressure of lipid monolayers (157, 158) and to tend to reverse the phases of oil in water emulsions (155).

Two similar and simultaneously published theories are concerned with the formation of gas hydrates, forming structures called microcrystals or "icebergs" (143, 160). There is also a good correlation between the dissociation pressure of hydrates of gaseous anaesthetics and the anaesthetic pressure of the gas (143, 160). These gas hydrates are suggested to interact spatially with proteins or membranes, thus reducing the intracellular material flow and the electrical conductivity of the water. More recent investigations on animals (142) and man (141) show however that there is a reversibly increased permeability of membranes to cations during exposure to anaesthetic pressures of inert gases.

Besides the inert gas narcosis which has been studied on man and animals, there are also some reports on the effects of inert gases on microorganisms. The sensitivity pattern is very much like that of anaesthesia. The heavier the gas and the higher the partial pressure of gas, the more pronounced is this effect. The growth rate of Neurospora crassa was retarded in this way by the noble gases (145) as were HeLa cells (144). During exposure of Neurospora crassa to helium, krypton, and xenon it was shown that xenon inhibited the cytoplasmic streaming effectively while krypton was less efficient and helium had no effect at all (146). Investigations with anaerobically growing bacteria with corresponding sensitivity pattern to inert gases show that it was not merely a matter of interaction, e.g. spatial competition, with oxygen (150). However, striking evidences have been found that such competition may also take part in some situations. The reduction of the oxygen dependent radiosensitivity of tissues from Vicia faba and Shigella flexneri by inert gases was fairly well correlated to their anaesthetic power which the investigators attributed to the displacement of oxygen from a specific site within the cell (151).

Several enzymes have also proved to be sensitive to high pressure if inert gases (153, 152).

In the series of noble gases helium is the lightest one and it has seldom shown any inhibitory or anaesthetic effect. As a matter of fact it sometimes seems to promote slightly some microbial activities (150, 147, 149).

This investigation was undertaken in order to see if the previous findings, i.e. that argon but not helium is inhibiting to spore germination, might be related to the earlier reported effects of inert gases on life reactions. If so, then a more pronounced effect might be expected if the heavier gases krypton and xenon were used.

Materials and methods

Spores. Spores of Bacillus cereus (SIK No. 216) were prepared as described on page 70.

Gases. Krypton and xenon with purity 99.997% were purchased from Alfa AB, Malmö. The largest impurities were nitrogen and xenon and nitrogen and krypton respectively.

Pressure treatment. 16 ml of washed spore suspension in 0.067 M phosphate buffer (pH 7.0, $OD^{620} \sim 0.8$) was pipetted into a polished stainless steel (SIS 2343) tube with inner diameter 16 mm and length 180 mm (Fig. 1). Both ends were furnished with stainless steel valves. Most of the air was removed by vacuum suction to about 50 mm Hg and the gas was then filled into the tube to the desired pressure. The sample was allowed to equilibrate with the gas phase for one hour, during this time it was frequently shaken.

The germination was initiated by addition of adenosine and L-alanine dissolved in the same buffer. The final concentrations were 2 mM and 10 mM respectively. The concentrated solution of the initiators were pipetted into a second tube connected above the sample tube and the germination was started by the opening of the upper valve (Fig. 1). After one minute of mixing a sample was taken, aerated vigorously in a vortex for 30 seconds, and then placed in a photometer with a recorder. Then germination was measured as the decrease in optical density. Five or six samples were taken in this way during one experiment.

The germination status at the moment of sampling from the pressure vessel was calculated from the decrease in optical density as compared with the optical density of the first sample. In each case this germination was verified by microscopic examination.

Temperature. The temperature in all experiments was $25 \pm 1^{\circ}\text{C}$.

Calculation of germination inhibition. Before each experiment some of the spore suspension was furnished with germination initiators and the germination was recorded. This sample is referred to as control.

The germination curve was calculated as

$$1 - \frac{\text{OD}_t}{\text{OD}_i} = f(t)$$

where OD_t and OD_i are the optical density at 620 nm at time t and zero respectively. This function was plotted for each germination experiment, and compared to the corresponding function from the control.

Results and discussion

The inhibitory effect of xenon and krypton on the germination of B. cereus spores is evident from fig 2 and 3. Since spores from different batches did not germinate identically a zone covering the 17 controls made in this investigation has been used in these figures. It seems as if half an atmosphere of xenon has no effect while one atmosphere of xenon is slightly inhibitory. At 4 and 6 atm the inhibition was complete at least during 2 hours. Krypton exerts a similar but somewhat weaker inhibitory power.

When the inhibited spores were brought out from the pressure vessel they started to germinate. This subsequent germination sometimes proceeded like the germination of the control (fig. 4). In some experiments however the subsequent germination was slower, especially in samples which had been inhibited for longer times (fig. 5 and 6).

In the previously described investigation on inhibition with nitrogen, argon and carbon dioxide it was a common, but not ultimate, experience that spores which had been inhibited for a long time gradually lost their capability to germinate when transferred into air. This type of inhibited germination was reversed when more germination initiators were added. The same events occurred when

germination initiators were added to krypton inhibited spores which were not able to germinate completely during the subsequent air incubation (fig. 6). The material in this investigation is limited because of the high costs of these gases but the tendency in 6 experiments with xenon inhibition was that most subsequent germinations proceeded like the control while in 4 experiments with krypton inhibition there was always a tendency to slower germination on the transfer into air.

The recovered ability to germinate on the transfer into air raises the question whether oxygen deficiency is involved in the inhibition. Special attention has been paid to elucidate this. Firstly there is no evidence that oxygen is needed for germination of spores, not even for spores from obligately aerobic species (196). As an extra control concerning the very strain of Bacillus that was used in this investigation an experiment was undertaken in a typical anaerobic medium, viz. nitrogen flushed buffer with 0.1% sodium thioglycollate. No inhibitory effect was found in this experiment (fig. 7). Secondly there was no inhibition in the previous helium experiments, and if inhibition was caused by oxygen deficiency in the other experiments then increased total gas pressure should yield less and not more inhibition. Furthermore no attempts were made in this investigation to eliminate all the oxygen. The germination medium, which constituted one fifth of the sample volume, was air saturated which caused the test solution to contain at least 20% of the normal amount of oxygen.

Experiments on the effects of inert gases on oxygen dependent radiosensitivity (151) supports the hypotheses that there might be a competition for space on specific sites. If this were the case high gas pressure might yet inhibit a hypothetically oxygen dependent germination. Therefore experiments were undertaken with 5 atm of krypton and 1 atm of air. This atmosphere caused the same inhibition as that of just 5 atm of krypton (fig. 3). These facts and the previous results that air at 100 atm exerts almost exactly the same inhibitory effect as does nitrogen at 100 atm, eliminates the possibility that the gas inhibited germination is a consequence of displacement of oxygen.

But the question why these gases inhibit the germination of spores remains. The inhibitory potency can be arranged according to $\text{He} < \text{N}_2 = \text{air} < \text{Ar} < \text{Kr} < \text{Xe} < \text{N}_2\text{O} < \text{CO}_2$ (table I). This is fairly well correlated to the anaesthetic potency of these gases (fig. 8). These circumstances and the fact that the biological effect of these gases is not specifically directed towards higher organisms but is rather a common life retarding process acting on any organism tested from bacteria to man, makes it plausible to assume a common mechanism lies behind.

At the moment one can only speculate in how the spore germination is inhibited since the problem involves two sets of reactions, none of which are fully understood. Firstly, what type of reaction does the gases exert on the molecular level, and secondly, what is the mechanism behind the initiation of germination of the spore?

There is one process that seems to dominate in many theories behind both these phenomena - the permeability. Theories on inert gas anaesthesia deal mostly with changes in permeability within the cell, regardless as to whether this is caused by gas hydrates changing the water structure or by the solution of the gases in membranes. Direct microscopic observations of hindered cytoplasmic streamings emphasizes the importance of inhibited transports as a consequence of inert gas exposure (146). The initiation of germination of spores is also known to involve the onset of transport mechanisms for ions and molecules (216) which emphasizes the importance of permeability changes in the spore.

The mechanism behind the inhibition of spore germination might be a hindered transport process within the spore. Gould and Sale (67) have suggested that the enhancement of germination by hydrostatic pressure is caused by an increase in the permeability of the spore to the germination initiator. The opposite effect of inert gases on germination might be explained by a corresponding blockade of the penetration of the initiator into the spore. The many evidences and hypothesis that hint at a reduction in cellular permeability of water dissolved compounds caused by inert gases support this idea.

However, the gas mediated inhibition after long incubation time remains when the inhibitory agent is removed and the addition of more germination initiator restores the germination. These circumstances and the fact that neither the spores per se nor the germination initiators were affected by the gases also suggest that the inert gas exposure causes an effect of the binding of the initiators to the spore.

TABLE I.

GAS	P_A (ATM)	P_I (ATM)
He	> 100	> 100
N ₂	29	40 - 50
Ar	20	5
Kr	2.9	3 - 5
Xe	0.85	1
N ₂ O	0.90	0.5 - 1
CO ₂	0.25	0.2 - 0.5

COMPARISON BETWEEN THE ANAESTHETIC PRESSURE (P_A) ACCORDING TO REF 143 AND THE LOWEST PRESSURE (P_I) AT WHICH INHIBITION OF THE GERMINATION OF B. CEREUS SPORES HAS BEEN FOUND IN THIS THESIS.

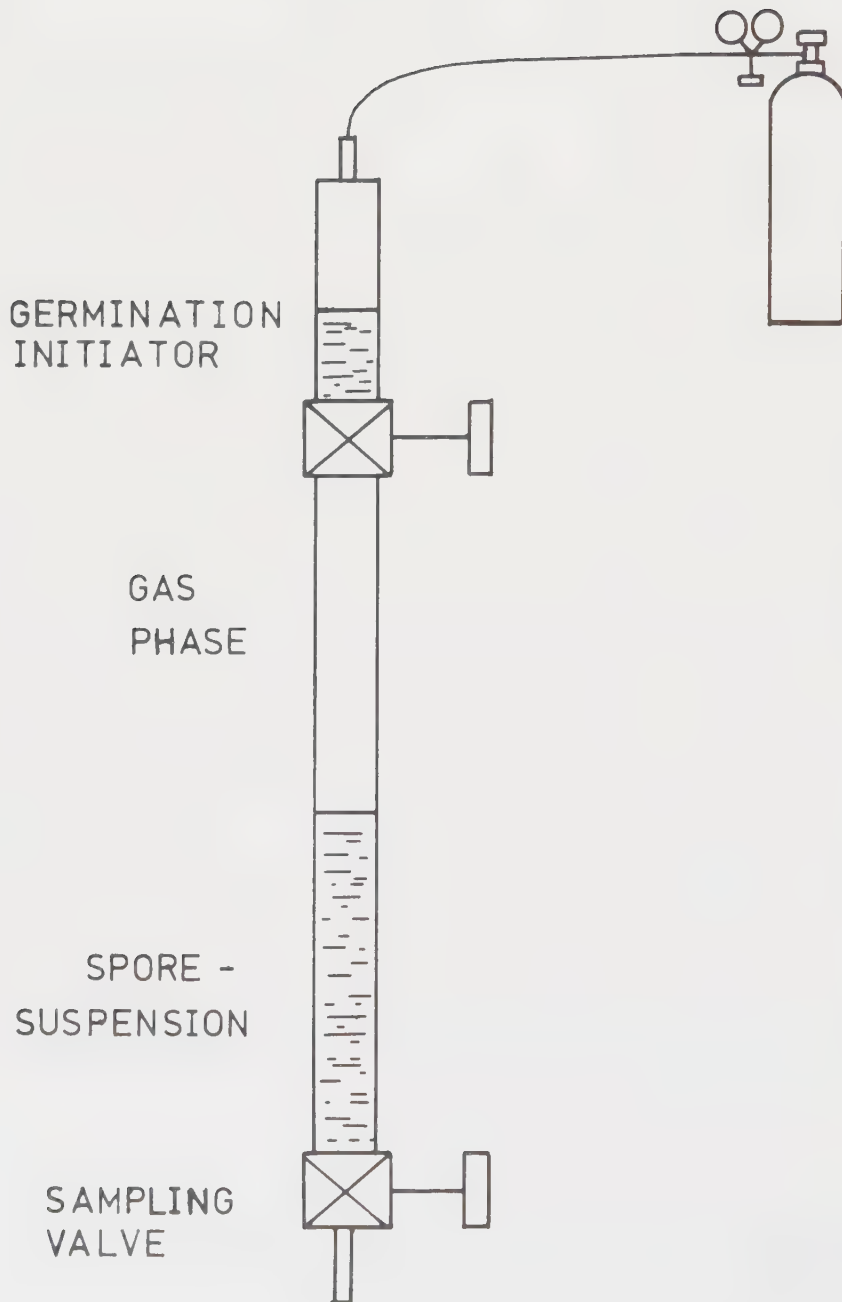


FIG 1. EQUIPMENT FOR EXPOSURE OF SPORES TO HIGH GASPRESSURE DURING GERMINATION.

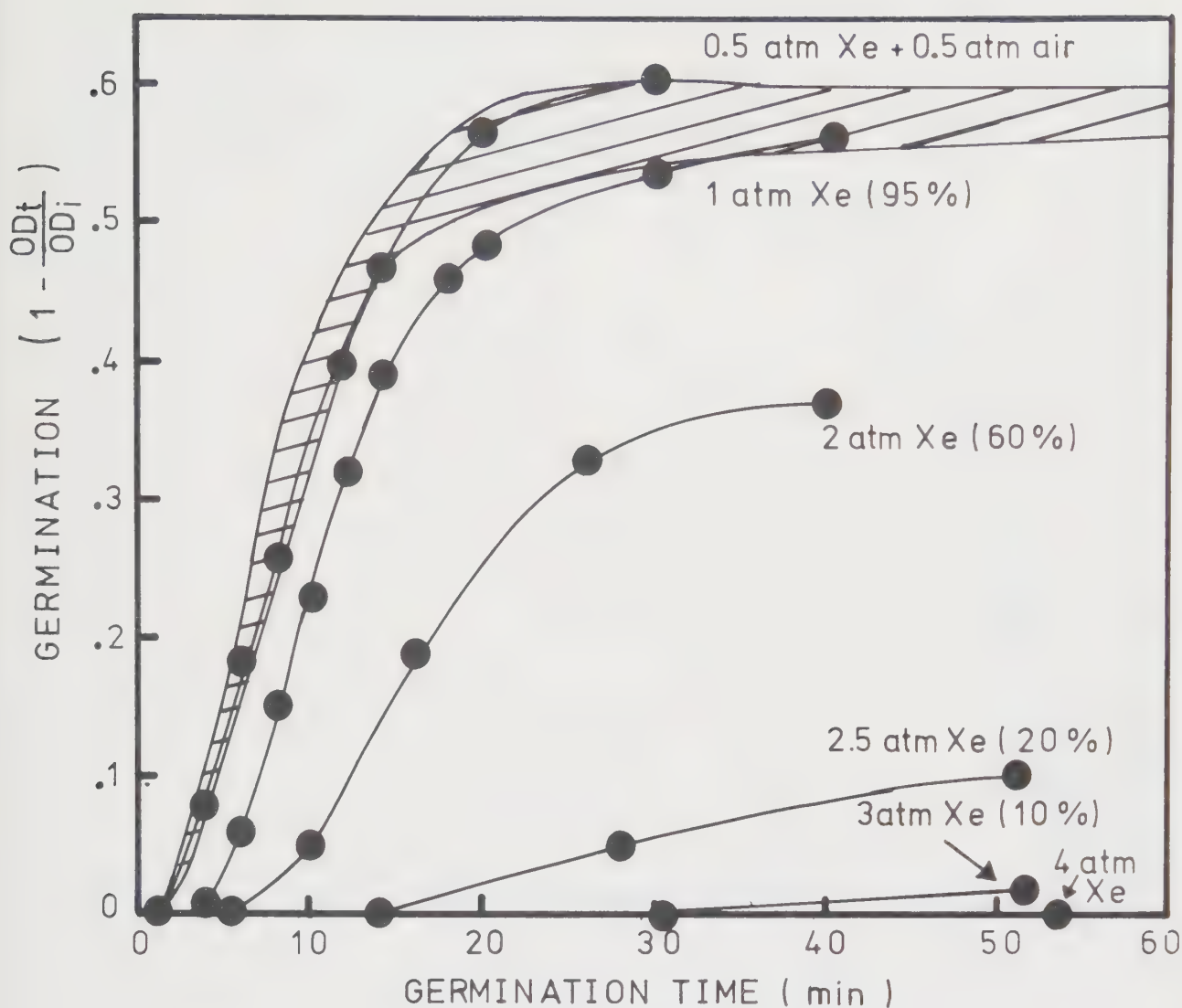


FIG 2. EFFECT OF XENON ON THE GERMINATION OF *B. CEREUS* SPORES. THE DASHED AREA COVERS ALL CONTROL GERMINATIONS IN 1 ATM OF AIR. THE FIGURES IN BRACKETS GIVE THE PERCENTAGE OF GERMINATED SPORES ACCORDING TO MICROSCOPIC OBSERVATION OF THE LAST SAMPLE IN THE CURVE.

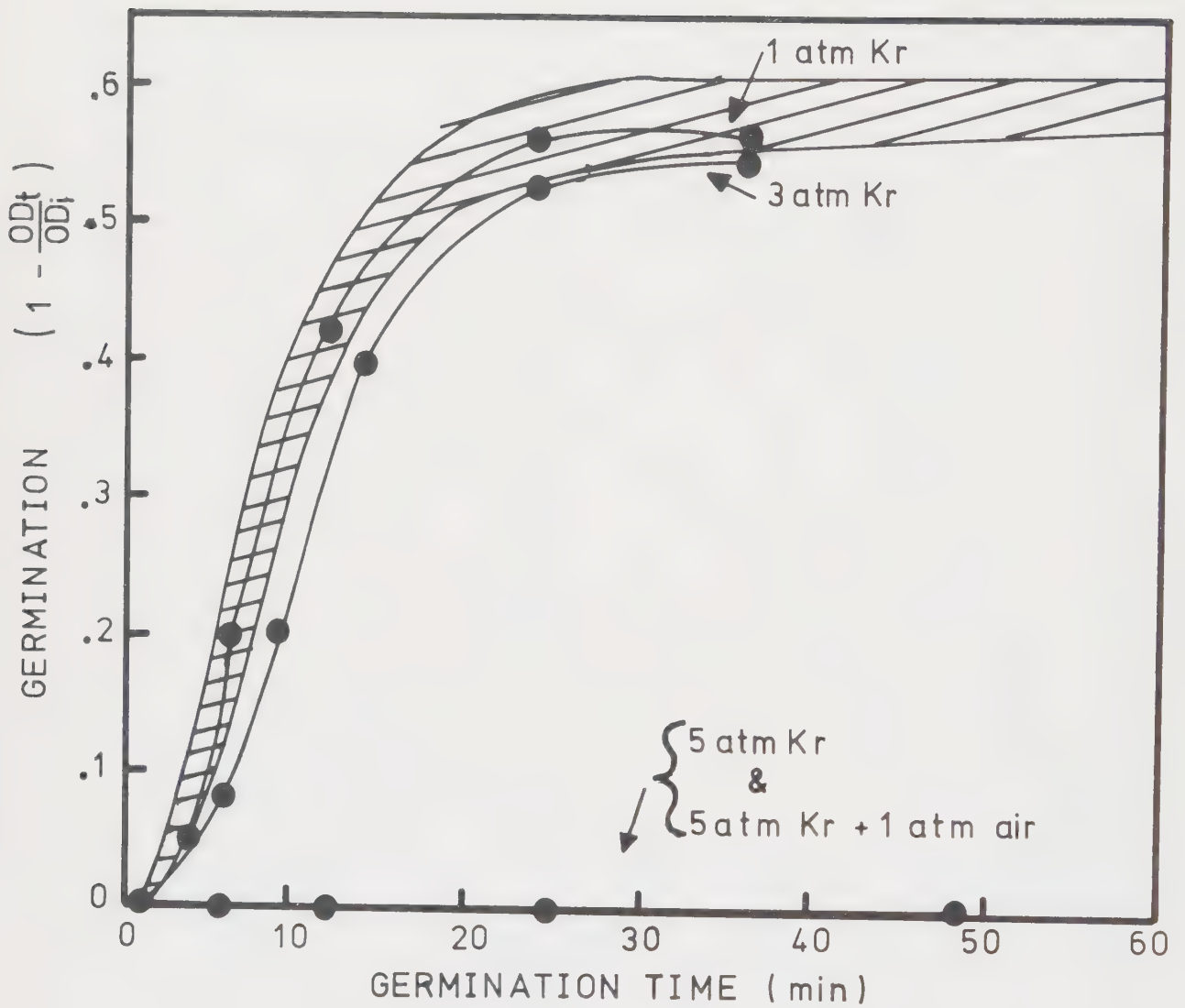


FIG 3. EFFECT OF KRYPTON ON THE GERMINATION OF *B. CEREUS* SPORES.
THE DASHED AREA COVERS ALL CONTROL GERMINATIONS IN 1 ATM OF AIR.

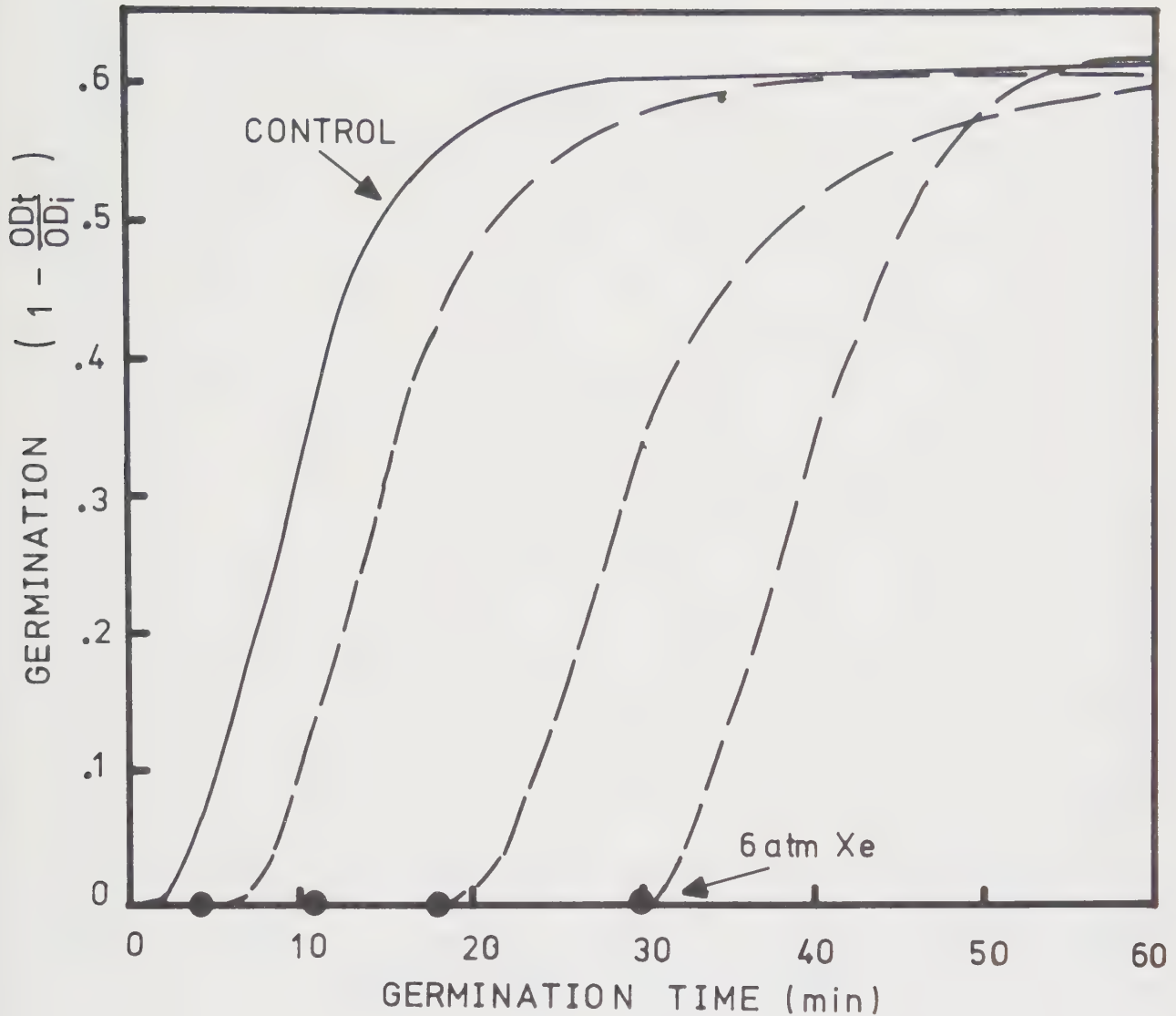


FIG 4. AN EXAMPLE OF COMPLETE REVERSIBILITY OF XENON-INHIBITED GERMINATION OF *B. CEREUS* SPORES. THE SPORES WERE SUBJECTED TO 6 ATM OF Xe, WHICH INHIBITED THE GERMINATION COMPLETELY. THE POINTS INDICATE THE STATUS OF THE GERMINATION IN 6 ATM OF Xe, AND THE DASHED LINES SHOW THE GERMINATION OF THE SAMPLES AFTER REMOVAL OF MOST OF THE XENON BY AERATION.

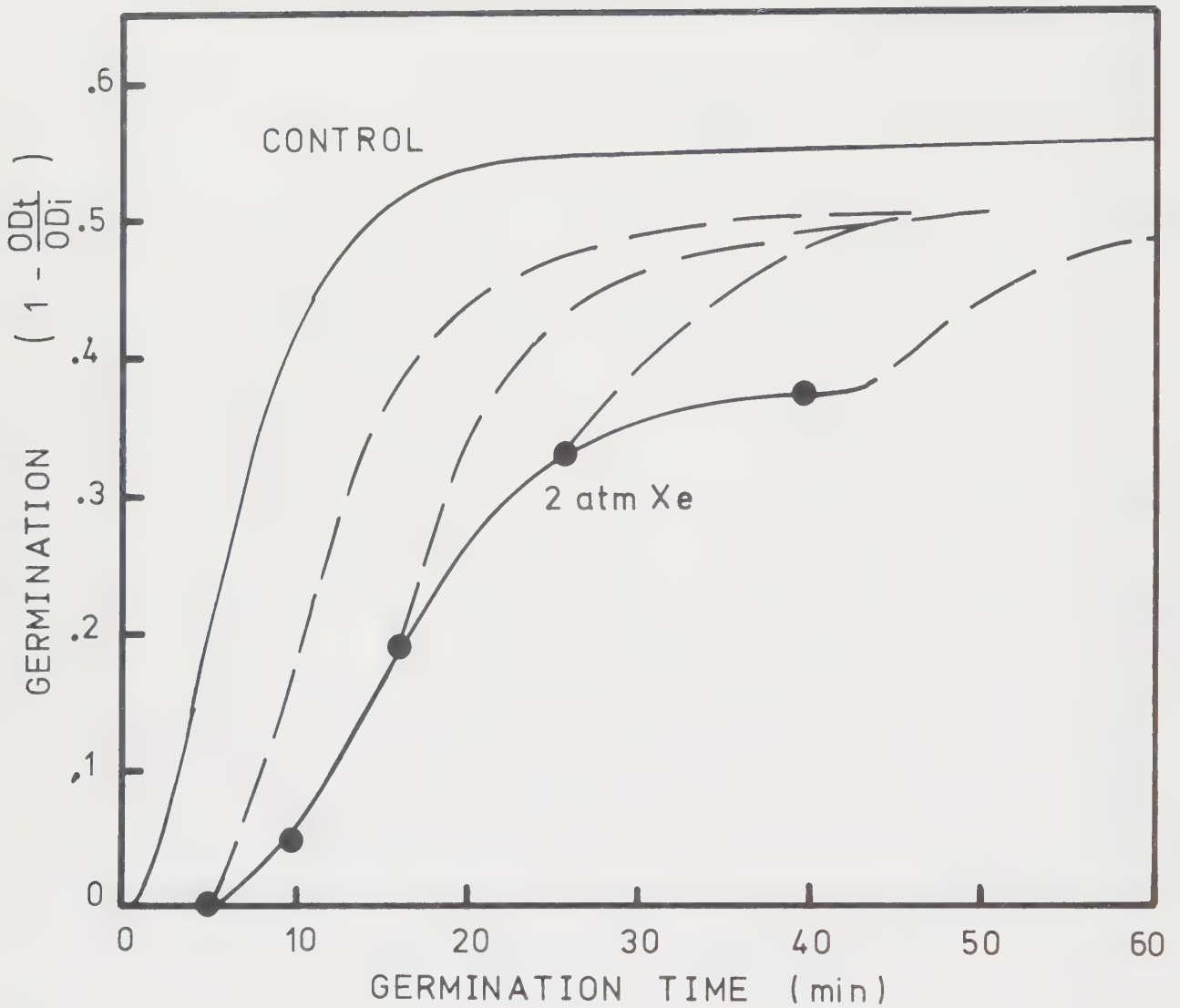


FIG 5. AN EXAMPLE OF NON-COMPLETE REVERSIBILITY OF XENON INHIBITED GERMINATION OF B. CEREUS SPORES, WHICH WERE INHIBITED BY 2 ATM OF Xe. THE POINTS INDICATE THE STATUS OF THE GERMINATION IN 2 ATM OF Xe, AND THE DASHED LINES SHOW THE GERMINATION OF THE SAMPLES AFTER REMOVAL OF MOST OF THE XENON BY AERATION.

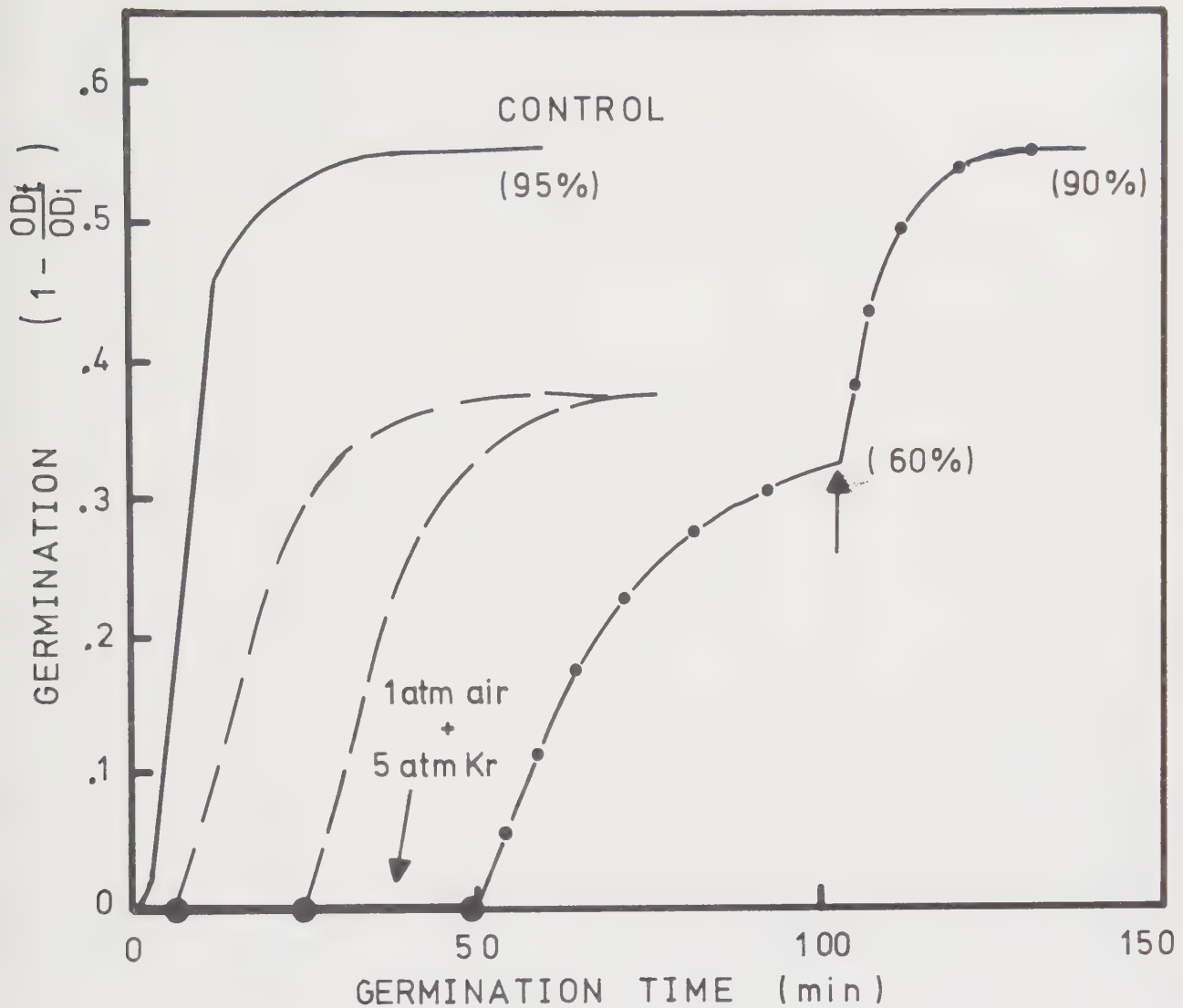


FIG 6. REVERSAL OF KRYPTON INHIBITED GERMINATION OF B. CEREUS SPORES. THE LARGE POINTS INDICATE THE STATUS OF THE GEMINATION IN 5 ATM OF KR + 1 ATM OF AIR. THE DASHED LINES SHOW THE GERMINATION OF AERATED SAMPLES. AT 50 MIN THE TOTAL GAS PRESSURE OF THE VESSEL WAS REDUCED TO 1 ATM. THEN ABOUT 60% OF THE SPORES GERMINATED. AT THE ARROW MORE GERMINATION INITIATOR WAS ADDED. THE GERMINATION THEN CONTINUED TO ABOUT 90% ACCORDING TO MICROSCOPIC OBSERVATION.

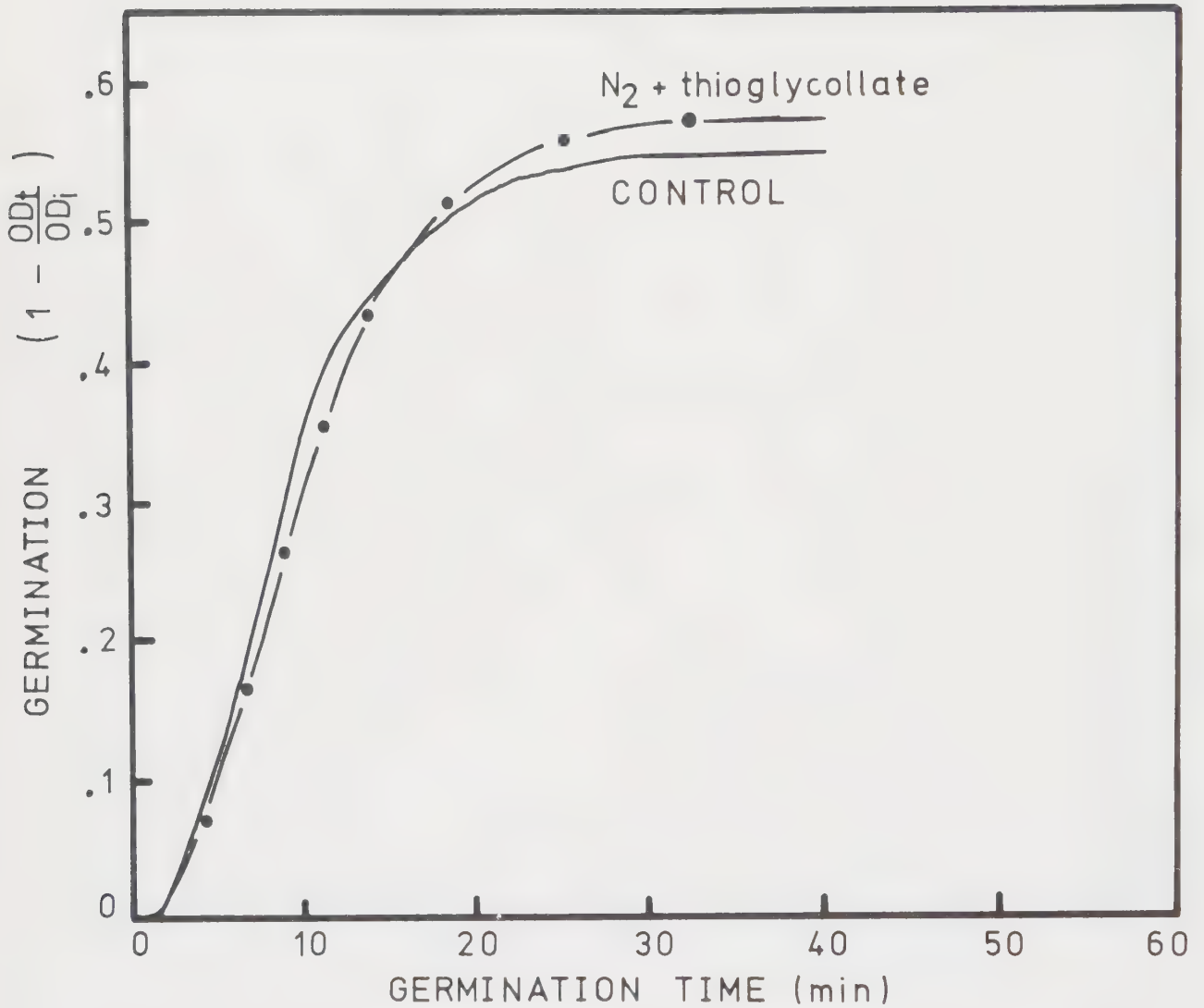


FIG 7. THE EFFECT OF LOW OXYGEN TENSION ON THE GERMINATION OF *B. CEREUS* SPORES. THE CONTROL WAS GERMINATED IN 1 ATM OF AIR, AND THE OTHER SAMPLE WAS DEGASSED WITH NITROGEN, AFTER WHICH 0.1% SODIUM THIOGLYCOLLATE WAS ADDED.

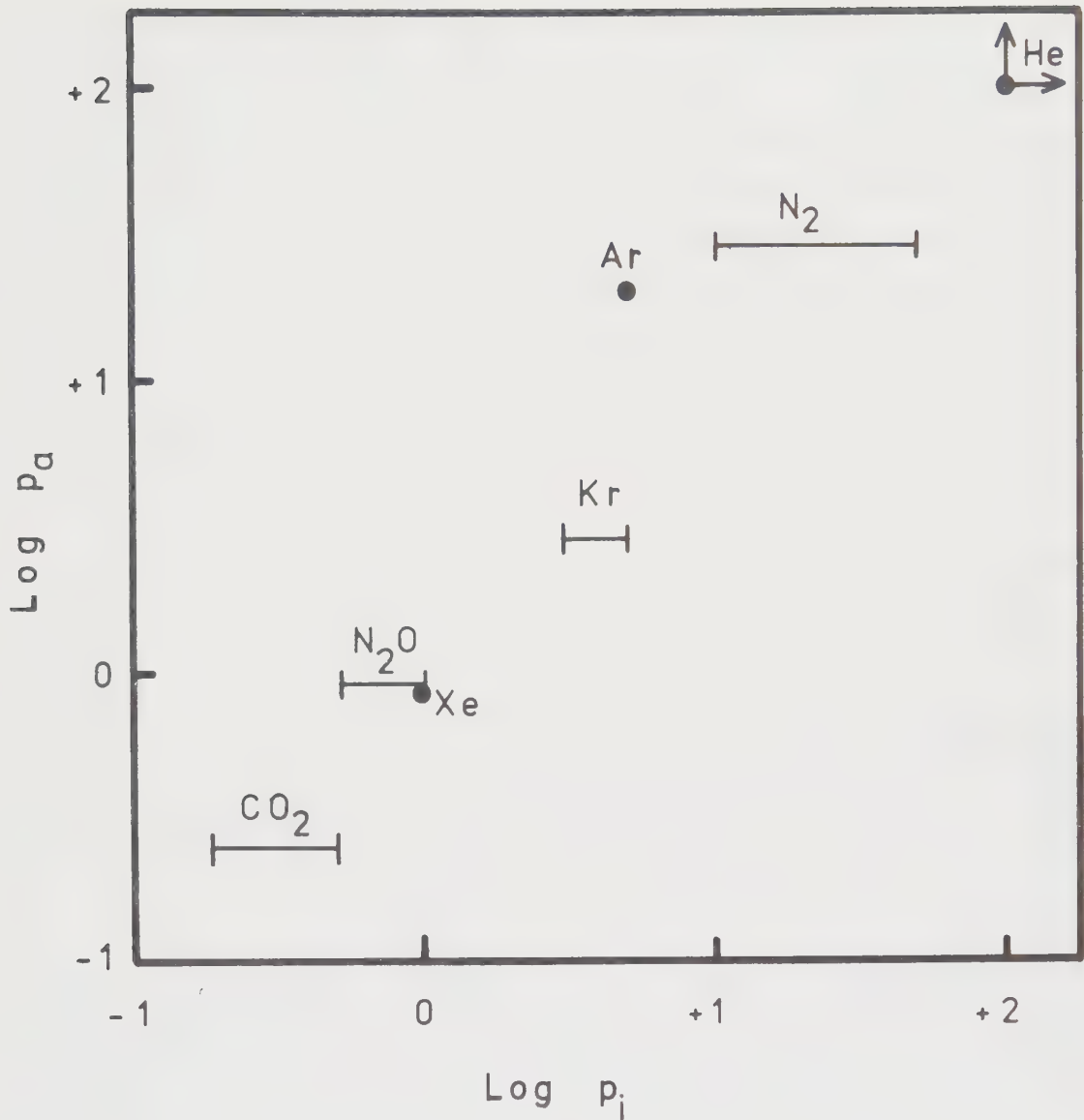


FIG 8. A COMPARISON BETWEEN THE ANAESTHETIC PRESSURE OF SOME GASES ACCORDING TO REF 143, (P_A), AND THE LOWEST PRESSURE AT WHICH INHIBITION OF SPORE GERMINATION IS EVIDENT ACCORDING TO THIS THESIS, (P_I). THE PRESSURES ARE GIVEN IN TABLE I. NO INHIBITION WAS FOUND BY 100 ATM OF HE AND NO ANAESTHETIC EFFECT HAS BEEN FOUND BY THIS GAS.

GENERAL DISCUSSION

This work on effects of high pressure of gases on microbial processes was started as an approach to a single cell protein project based on microbial conversion of methane to biomass. It was suspected that the solubility of methane in water was critically low. This problem was further extended by the fact that bacterial methane oxidation is a very oxygen consuming process which means that a large amount of the gas phase must contain oxygen.

Experiments with a mixed culture of methane oxidizing bacteria revealed that maximum growth rate was not achieved until the partial pressure of methane was increased to about one atm. These results were further supported by the detection that enrichment cultures grew faster in 5.5 atm of methane than in 0.5 atm of methane. The growth promoting effect of high methane pressure was used as a tool in isolation experiments.

Increased gas pressure over a microbial culture influences the growth conditions in yet another way. It provides an increased gas transfer rate. However, the technique includes some problems: the toxicity of oxygen at high pressure and the less drastic effects of other gases like carbon dioxide and possibly also by inert gases like nitrogen.

During the first years of this work the single cell protein project gradually turned over to production based on methanol. The high pressure technique offered interesting aspects also in such a process, e.g. the effect on gas transfer rate and the fact that a high air lift fermenter must partly operate under considerable pressure. Therefore this work was focused on the effects of high air pressure on microbial processes in general. Experiments were performed to elucidate the effect of high air pressure on some basic microbial processes: growth, sporulation and spore germination. It was found that high oxygen pressure inhibits sporulation as well as growth of B. stearothermophilus and B. cereus.

These investigations suffered from the same lack of precision as did many similar experiments described in literature, the reason being that suitable equipment for cultivation of microorganisms under high pressure during

controlled conditions did not exist. Therefore, the design of a system for this type of experiment was undertaken.

The high pressure fermenter that was designed offers facilities for the same degree of control of the process as does an ordinary fermenter. The capacity of the fermenter is 2 - 4 litre of culture at 0 - 15 atm gauge pressure. The fermenter is designed as a standard fermenter but the dosing pumps and reservoirs for liquids and the electrodes are built into the fermenter. All electrical signals are transferred through special pressure tight connectors. The function of the pressure fermenter and its control system was tested in production of bacterial biomass from methanol. This system offers extended possibilities in the research of effects of high gas pressure on microbial processes.

During the experiments concerning the germination of bacterial spores under high air pressure it was noticed that this process was very resistant to oxygen since it was not affected by 10 atm of air and only slightly retarded by 50 atm of air. When control experiments were performed it was found that nitrogen was as inhibitory as air for the germination. This interesting and rather unexpected phenomenon was investigated further. It was found that many gases inhibited the germination of B. cereus.

The potency of inhibition could be arranged $\text{He} < \text{N}_2 = \text{air} < \text{Ar} < \text{Kr} < \text{Xe} < \text{N}_2\text{O} < \text{CO}_2$. The more potent the gas, the lower the pressure needed to visualize the effect. Helium did not inhibit the germination at 100 atm and the pressure limit for start of xenon inhibition was about 1 atm. The inhibitory potency of the gases was compared to their anaesthetic effects and a good correlation was found. As a matter of fact, the exact pressure of the gas at which inhibition of germination started was roughly the same as the anaesthetic pressure of the gas. It was suggested that there might be a common mechanism in behind these reactions.

The wide gap between the evolutionary stage of the biological reactions that are influenced by the gases makes perhaps the suggestion seem a little hazardous, but the fact that the same gases, with a similar potency, have been reported to exert an inhibitory effect on growth of microorganisms as well as human cells, strongly supports the idea that the basis of this phe-

nomenon is to be looked for on a basic molecular level in the cell. Several theories on the effect of inert gases on cellular reactions are concerned with an effect of the gas on membrane permeability. It is supposed in literature that the gases become enriched in lipid fractions, e.g. membranes, of the cell and that this enrichment causes a swelling of the membrane which alters its properties.

Hydrostatic pressure has earlier been suggested to cause an increased permeability for certain vital compounds within the spore, which accelerates the germination process. The action of high pressure of gases is now suggested to be a contrary mechanism in which the dissolution of gases in the spore membranes cause a decreased permeability to these vital compounds within the spore.

Earlier findings that bicarbonate exerts an inhibitory effect on spore germination was verified in this work and the mechanism of this inhibition is discussed in context with the effects of the gases and it was then suggested that bicarbonate inhibits the germination by the action of free carbon dioxide that is generated in a bicarbonate solution.

SUMMARY OF RESULTS

A mixed culture of methane oxidizing bacteria was cultivated at 0-3 atm of methane and at 0-2.8 atm of oxygen. The growth rate increased with increasing methane pressure to about 1 atm. K_s was calculated to 0.08 atm. Oxygen pressure exceeding 0.2 atm decreased the growth rate. At 2.8 atm of oxygen no growth commenced and this pressure injured the culture so that the subsequent growth after pressure release was considerably slower than the growth of a control.

The growth of this culture on silica gel was very sparse as compared to growth on agar plates. When the silica plates were incubated in 5.5 atm of methane some colonies grew thicker than the control in 0.5 atm of methane. A pure strain which could grow on methane was isolated. This strain was not able to grow on methanol or nutrient broth. However, after repeated reinoculation in liquid cultures it lost the ability to grow on methane.

Growth of the mixed culture on agar was more rich than growth on silica gel. When such plates were incubated in air there was still a considerable growth. Corresponding growth on agarose plates in air was not so rich but still evident.

Enrichment cultures which were incubated in 5.5 atm of methane grew faster than corresponding controls in 0.5 atm of methane. However, the distribution of different types of organisms in these cultures was not influenced by the pressure incubation.

The growth rate of E. coli was not affected by 4 or 6 atm of air. However, these pressures caused an evident lag phase, which was dependent on an initial decrease in the viable count of the pressure incubated cultures.

Growth of B. stearothermophilus was inhibited at air pressures above 3 atm. At 7 atm of air no growth occurred. The organism did not adapt to growth at increased air pressure.

Growth and sporulation of B. cereus was inhibited at air pressures exceeding 2 atm. The growth recovered immediately after pressure release but the subsequent sporulation was less efficient than that of the control.

A fully instrumented high pressure fermenter for 4-litre cultures at pressures up to 15 atm gauge pressure was developed.

Germination of *B. cereus* spores was slightly inhibited by 50 atm of air. The oxygen pressure of this atmosphere was not the inhibiting agent. Helium at 100 atm did not influence the germination kinetics. Nitrogen exerted the same inhibition as did air. Argon showed a stronger effect. The inhibition started at 5 atm and was complete at 50 atm. Krypton started to inhibit the germination at 3-5 atm, xenon at 1 atm, nitrous oxide at 0.5-1 atm and carbon dioxide at 0.2-0.5 atm. Bicarbonate caused an inhibition which was related to the actual concentration of free carbon dioxide in the sample.

These inhibitions were reversible if the inhibiting agent was removed within a certain time (hours). After longer exposures to the inhibiting atmosphere the germination did not recover when the gas was removed. However the addition of more germination initiator (L-alanine and adenosine) then restored the germination.

Exposure of non-germinating spores to these atmospheres during 24 hours did not influence the germination kinetics of the spores when they were initiated to germinate after the treatment.

Exposure of the germination initiators to germination inhibiting atmospheres did not influence their ability to initiate the germination after the exposure.

It was suggested that the gases inhibited the germination by influencing the permeability of the spore.

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